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(57) Abstract

Methods are provided for identifying agents useful for decreasing undesired cell proliferation, e.g., to treat a neoplasm. Methods are also provided for identifying agents useful for inducing the replication of cells in a terminally differentiated state in order to, e.g., regenerate a damaged tissue, e.g., muscle, neural, or epithelial, or hematopoietic tissue. The agents useful in the invention either enhance or decrease the interaction between a pocket protein, e.g., retinoblastoma protein, and a tissue-specific transcription factor, e.g., members of the MyoD, MEF2, or MASH families of transcription factors.

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CONTROL OF CELL PROLIFERATION

Background of the Invention

The invention relates to screening procedures that identify agents for decreasing the proliferation of neoplastic cells, and agents for inducing the replication of cells in a terminally differentiated state. The invention also relates to the use of such agents as therapeutics.

An essential step in the differentiation pathway of many cell types is their permanent withdrawal from the cell growth cycle, a pre-requisite for the acquisition of their terminally differentiated phenotype. Terminally differentiated cells can remain viable for many decades despite their inability to respond to growth factor stimulation with events that lead to cell division.

Terminal differentiation of many cell types is characterized by two main events: irreversible withdrawal from the cell cycle and biochemical differentiation. Cultured skeletal myogenic cells provide a good experimental system to study this process. During terminal differentiation, mononucleated myoblasts cease DNA synthesis, irreversibly withdraw from the cell cycle (commitment), and fuse to form multinucleated myotubes. Concomitant with these cellular events, a battery of muscle-specific genes that includes contractile as well as regulatory proteins is induced (biochemical differentiation). Both biochemical differentiation and commitment are required for the maintenance of the terminally differentiated state since biochemically differentiated but uncommitted cells can re-enter the cell cycle and de-differentiate in response to appropriate growth stimuli (Taubman et al., *J. Cell. Biol.* 108:1799-1806, 1989). However, committed terminally differentiated skeletal muscle cells do not

reinitiate DNA synthesis or mitosis, even in response to serum or growth factor stimulation (Konigsberg et al., *J. Biophys. Biochem. Cytol.* 8:333-343, 1960; Stockdale et al., *Exp. Cell. Res.* 24:508-520, 1961; Nadal-Ginard, 1978; Endo et al., *Mol. Cell. Biol.* 6:1412-1421, 1986). It has been suggested that the lack of growth response to mitogen stimulation is due to the loss of the appropriate membrane receptors. This conclusion is not supported by the kinetics of receptor loss or by the fact that terminally differentiated myotubes exhibit robust responses in the induction of immediate early genes in response to growth factors that are required for myoblast growth. These cells, however, are unable to re-initiate DNA replication (Endo and Nadal-Ginard 1986 supra).

Certain DNA and RNA tumor viruses including simian virus 40 (SV40), polyomavirus (Py) and adenovirus E1A are able to induce host cell DNA synthesis and mitosis in contact-inhibited cells and X-irradiated cells. These viruses have been used in the past to determine whether transformation of myogenic cells inhibits their differentiation and/or can induce DNA synthesis and mitosis in terminally differentiated myotubes. In many instances the results obtained have been ambiguous or contradictory. Myoblasts infected with SV40 or Py retained their ability to differentiate early after infection (Fogel et al., *Proc. Natl. Acad. Sci. USA* 58:967-973, 1967; Yaffe et al., *Nature* 215:421-424, 1967), but this ability was lost by further subculturing (Holtzer et al., *Proc. Natl. Acad. Sci. USA* 72:4051-4055, 1975). DNA synthesis and some mitotic figures were detected at the time of fusion of the infected myoblasts into myotubes (Fogel et al. 1967 supra; Yaffe et al. 1967 supra; Graessmann et al., *Dev. Biol.* 35:180-186, 1973).

Retinoblastoma (pRB) is a nuclear phosphoprotein and tumor suppressor whose antiproliferative activity is

regulated by phosphorylation/dephosphorylation events that take place at specific phases of the cell cycle (DeCaprio et al., *Cell* 58:1085-1095, 1989; Buchkovich et al., *Cell* 58:1097-1105, 1989). It is thought that pRB is phosphorylated by a member of the cdc2 family of serine-threonine kinases. In eukaryotic cells the cdc2 kinases regulate cell cycle progression from the G1 to S phase, as well as from the G2 to M phase. The kinase activity is regulated in turn by the association of its catalytic subunit with different kinds of regulatory subunits, the cyclins, which are synthesized and degraded at different phases of the cell cycle. The association of cdc2 with each cell cycle-specific cyclin is thought to confer substrate specificity to the kinase.

The ability of SV40, polyoma and adeno viruses to inhibit the terminal differentiation of myogenic primary and established cells is a well-documented observation (Fogel et al. 1967 supra); Yaffe et al. 1967 supra; Graessmann et al. 1973 supra; Holtzer et al. 1975 supra; Endo and Nadal-Ginard, *Cell. and Mol. Biol. of Muscle Dev.*, Kedes and Stockdale, Eds. (A.R. Liss, Inc.) pp. 95-104, 1989). The oncoproteins of these three viruses, in addition to human papilloma virus E7 protein, have the common property of binding to the pocket domain of unphosphorylated pRB, thereby inactivating the growth suppressive function of pRB (DeCaprio et al., *Cell* 54:275-283, 1988; Whyte et al., *Nature* 334:124-127, 1988; Dyson et al., *Science* 243:934-940, 1989; Dyson et al., *J. Virol.* 64:1353-1356, 1990).

Summary of the Invention

Applicants have identified a mechanism underlying the switch that determines whether a cell is in active growth phase, or whether it enters the terminally differentiated state. The switch is dependent on an

interaction between a pocket protein, such as pRB, and a cellular transcription factor. While the distribution of a pocket protein is ubiquitous, the distribution of transcription factors is unique to the tissue type for which the cell will be determined. For example, in muscle cells these transcription factors are exemplified by members of the MyoD family and the MEF2 family, and in nerve cells by members of the MASH family of transcription factors. Without binding to the pocket protein, tissue-specific transcription factors are not able to turn on or enhance transcription of tissue-specific genes. Similarly, without binding to the cellular factor, a pocket protein is unable to induce growth arrest. Thus for the first time, applicants have identified the mechanistic link between two aspects of terminal differentiation: commitment and biochemical differentiation.

Applicants' discovery provides the basis for screening therapeutic agents useful for regulating the switch between the cell's growth phase and a terminally differentiated state. This provides long awaited advantages in two areas. The first advantage is to identify agents that enhance the interaction between a pocket protein and a transcription factor. These agents can be used to stop undesired cell proliferation, e.g., the proliferation of a neoplasm. Neoplastic cells are induced by these agents to irreversibly enter a terminally differentiated state, thereby stopping the growth of, and decreasing the size of, the neoplasm. The second advantage is to identify agents that can be used to treat damaged tissue. For instance, cells of a damaged heart or nerve can be treated with an agent that reduces the interaction of a pocket protein and a transcription factor, thereby inducing cells of that tissue to proliferate. This in effect regenerates the

tissue. As shown below, applicants have successfully induced terminally differentiated cells to proliferate.

In a first aspect, therefore, the invention generally features a method for identifying an agent that decreases undesired cell proliferation. The method involves (a) providing a system that includes a pocket protein and a factor that is required for terminal differentiation of a cell, the system being further characterized by conditions under which the pocket protein and the factor do not substantially interact; (b) providing the candidate agent in the system; and (c) measuring the interaction of the pocket protein with the factor, an increase in the interaction, relative to the interaction in the absence of the agent, indicating that the agent is capable of decreasing cell proliferation.

A "pocket protein" as used herein, refers to a member of a family of pocket proteins, generally characterized as being substantially homologous to pRb in the pocket domain, and/or in the transcription factor-binding domain described herein. Examples of pocket proteins include retinoblastoma protein (pRb), p107 (Ewen et al., *Cell* 66:1155-1164, 1991, hereby incorporated by reference), p300 (Mymryk et al., *Mol. Bio. Cell* 3:1107-1115, 1992, hereby incorporated by reference; Whyte et al., *Cell* 56:67-75, 1989, hereby incorporated by reference), or other proteins falling within this definition.

By "a factor," or "a factor that is required for terminal differentiation of a cell," is meant a cellular transcription factor, e.g., a protein, preferably a tissue-specific protein, without which the cell is not able to enter the terminally differentiated state. Examples of preferred factors include a member of the MyoD family, the MEF2 family, the MASH family, or the

RSRF family (Pollack et al., Genes Dev. 5:2327-2341; 1991). The MyoD family includes, for example, myogenin, myf5, and MRF4 (Olson, *Genes and Dev.*, 4:1454-1461, 1990). A member of the MEF2 family includes MEF2, aMEF2, xMEF2, hMEF2C, and hMEF2D (Yu et al., *Genes and Dev.* 6:1783-1798, 1992; for clarification of MEF2 family nomenclature see Table 1). A member of the MASH family includes MASH1 or MASH2 (Johnson et al., *Proc. Natl. Acad. Sci. USA* 89:3596-3600, 1992).

Table 1: Proposed MEF2 Nomenclature

Human Gene[1]	Products[2]	Previously Published Designations
hMEF2A	hMEF2A	RSRFC4, MEF2, SL-2(*Xenopus*) RSRFC9, aMEF2, a*MEF2
hMEF2B	hMEF2B	RSRFR2, xMEF2
hMEF2C	hMEF2C	hMEF2C, CMMEF2
hMEF2D	hMEF2D	Sl-1(*Xenopus*)

[1] h refers to human:A-D simply represents the order in which the corresponding cDNAs have been reported.

[2] Multiple alternatively spliced gene products designated collectively unless otherwise specified.

Candidate agents include a protein, e.g., a protein factor required for terminal differentiation of a cell; a polypeptide, e.g., a polypeptide, or polypeptide fragments that is substantially homologous to a portion of a pocket-binding domain of a transcription factor, or of a factor-binding domain of a pocket protein; an analog of one of the above proteins or polypeptides, e.g., an analog that functions as a competitive analog, a substrate analog, and/or as a transition state analog; a peptidomimetic compound (for a description of methods of designing peptidomimetic compounds, see *Tetrahadron Lett.*

32:4675-4678, 1991, hereby incorporated by reference; *Eur. J. Phar.* 205:1-6, 1991, hereby incorporated by reference; Life Sci. 49:1207-1212, 1991, hereby incorporated by reference); an organic compound; an inorganic compound; a nucleic acid fragment comprising a tissue-specific enhancer sequence. A "system", as used for screening these candidate agents, can be a cell; an extract, i.e., a cellular extract; an *in vitro* system; or an animal model, e.g., a transgenic non-human animal model. A "transgene" is defined as a piece of DNA which is inserted by artifice into a cell and becomes a part of the genome of the animal or tissue which develops from that cell. Such a transgene may be partly or entirely heterologous to the transgenic animal. A "transgenic animal" is an animal having cells that contain a transgene, which transgene was either a) introduced into the animal, or an ancestor of the animal, at an embryonic stage, or b) introduced into the somatic tissue of the animal.

"Undesired cell proliferation", as used herein, refers to uncontrolled reproduction of a population of cells, e.g., in an undesired location, or in a way that incurs undesired or harmful effects on the environment surrounding the cell, e.g., on an organism bearing the cell. Examples of undesired cell proliferation include, but are not limited to, any type of neoplasm, or to excessive growth of hematopoietic tissue, endothelial and smooth muscle tissue in the vascular wall, as well as other forms of hyperplasia. Where the neoplasm occurs in a tissue that is otherwise terminally differentiated, the candidate agent preferably affects the interaction between a pocket protein and a factor to restore the proliferating cell population to its original differentiated state.

In one preferred embodiment, the method of decreasing undesired cell proliferation further comprises: (d) providing a factor-specific deoxyribonucleic acid (DNA) sequence in the system, and (e) determining the binding between the factor and the DNA sequence, an increase in the binding, relative to the amount of the binding in the absence of the agent, indicating that the agent is capable of decreasing cell proliferation. In this context "binding" refers to a hydrogen bond association between the factor and the DNA sequence. A "factor-specific DNA sequence" as used herein refers to a DNA sequence of, e.g., 5-30, or 10-20, nucleotides that is specifically recognized by one of the above-listed cellular factors, e.g., a tissue-specific enhancer sequence.

In a second preferred embodiment to the method of decreasing undesired cell proliferation, wherein the system includes a proliferating cell line, or an extract of the cell line, the method further includes: (d) exposing the system to an antibody in the presence of the candidate agent, the antibody being one that is capable of precipitating a complex between the pocket protein and the factor; and (e) measuring the amount of the complex in the precipitate, an increase in the amount of the complex, relative to the amount of the complex precipitated from cells not exposed to the agent, indicating that the agent decreases cell proliferation. "Extract" refers to the intracellular contents of cells after removing the extracellular membrane. "Complex", as used herein, refers to an association of a first and a second component, e.g., a pocket protein and a factor. The association can include either or both covalent and noncovalent bonds. A "proliferating cell line" is a population of cells in a state of cell division and replication.

In another alternative embodiment to the method of decreasing undesired cell proliferation, the method further includes: (d) providing a reporter gene in the system, the expression of the reporter gene being dependent on the interaction of the pocket protein with the factor; and (e) measuring the level of expression of the reporter gene, an increase in the level, relative to the level of expression of the reporter gene in a system lacking the candidate agent, indicating that the candidate agent decreases cell proliferation. The system can be, but is not necessarily, a transgenic non-human mammal.

A "reporter gene" is a gene that produces a protein product that is easily measured in a routine assay. Suitable reporter genes known to those skilled in the art include chloramphenicol acetyl-transferase (CAT), luciferase, and beta-galactosidase. Convenient assays are, e.g., an enzymatic activity assay, a colorimetric assay, or a fluorometric assay. Additional examples of routine assays used to measure the concentration of a reporter gene product are provided in the examples below. Reporter genes useful in this invention are placed under the regulatory control of a promoter and/or enhancer sequence that is activated by a transcription factor, the transcription factor being part of a complex that also includes a pocket protein. The ability of the candidate agent to decrease cell proliferation is therefore indicated in this assay by an increase in the level of reporter gene expression, which is in turn a function of the ability of a pocket protein-transcription factor complex to bind the regulatory sequences upstream of the reporter gene. The "increase in the level of reporter gene product" is shown by comparing the measured amount of reporter gene product in a system exposed to the candidate agent relative to the level of reporter gene

product measured in a system that is not exposed to the candidate agent. This system experiences the same conditions (minus the candidate agent) as the system that is exposed to the candidate agent, and thereby serves as a negative control. The increased level of reporter gene product is generally 1-10 fold, preferably 10-100 fold, that of the level of reporter gene product of the negative control. An "enhancer region" is defined as a cis-acting DNA sequence capable of increasing transcription from a promoter that is located either upstream or downstream of the enhancer region. A "promoter region" is a cis-acting DNA sequence recognized by a complex comprising RNA polymerase for activation of transcription. Such DNA sequences are well known to those skilled in the art of eukaryotic gene expression.

The first aspect of the invention also includes a method for identifying an agent that increases the interaction between a pocket protein and a factor required for terminal differentiation of a cell. The method includes (a) providing a proliferating cell line; (b) allowing, e.g., by inducing, the cells of the cell line to differentiate in the presence of the candidate agent; and (c) measuring the extent of differentiation of the cells, an increase in the extent of the differentiation, relative to the extent of differentiation in cells not exposed to the agent, indicating that the agent increases the interaction. An "interaction" between a pocket protein and a transcription factor, as used herein, refers to the presence of the pocket protein and the transcription factor in the same protein complex, preferably involving a direct binding between the pocket protein and the transcription factor. In an *in vivo* system, the interaction is manifested by expression of tissue specific genes. By "increases the interaction" is meant

increasing either the proportion of components involved in the interaction, or strengthening the affinity of the binding interaction. In contrast, a "decrease in the interaction" means decreasing the strength of the interaction, inhibiting the formation of the interaction, or disrupting a previously existing interaction.

In a second aspect the invention generally includes a method for identifying an agent that induces a terminally-differentiated cell to proliferate. The method includes (a) providing a system that includes a pocket protein and a factor that is required for terminal differentiation of a cell, the system being further characterized by conditions under which the pocket protein and the factor are capable of substantially interacting; (b) providing the candidate agent in the system; and (c) determining the interaction of the pocket protein with the factor, a decrease of the interaction, relative to the interaction in the absence of the agent, indicating that the candidate agent is capable of inducing a terminally differentiated cell to proliferate. A "terminally differentiated cell", as used herein, refers to a cell, i.e., a cell in culture, or a cell in a tissue or organ, which has withdrawn from the cell cycle, and which is biochemically characterized by molecules typically expressed only in the tissue type of the particular cell. By "substantially interacting" is meant that a considerable fraction, e.g., 50%, preferably 60%, more preferably 80%, or most preferably 90-100%, of the pocket proteins and factors of the system are involved in an interaction with each other. By "proliferate" is meant at least two or more cycles of cell division and replication.

In one preferred embodiment of a second aspect of the invention, the method of inducing a terminally-differentiated cell to proliferate further includes: (d)

providing a factor-specific deoxyribonucleic acid (DNA) sequence in the system; and (e) determining the binding between the factor and the DNA sequence, a decrease in the binding, relative to the amount of the binding in the absence of the agent, indicating that the agent is capable of inducing a terminally differentiated cell to proliferate.

In a second preferred embodiment, wherein the system comprises a differentiated cell or cell line, or an extract of the cell or cell line, the method of inducing a terminally-differentiated cell to proliferate further includes: (d) exposing the system to an antibody in the presence of the candidate agent, the antibody being one that is capable of precipitating a complex between the pocket protein and the factor; and (e) measuring the amount of the complex in the precipitate, a decrease in the amount of the complex, relative to the amount of the complex precipitated from cells not exposed to the agent, indicating that the candidate agent is capable of inducing a terminally differentiated cell to proliferate.

In a third preferred embodiment of a method of inducing a terminally-differentiated cell to proliferate, the system is further characterized in being a differentiated cell line. The interaction is determined by measuring the extent of differentiation of cells of the cell line, a decrease in the extent of the differentiation, relative to the extent of the differentiation in cells not exposed to the candidate agent, indicating that the candidate agent is capable of inducing a terminally differentiated cell to proliferate. The "extent of differentiation" is a function of either the numerical proportion of cells that have entered the differentiated state, or the degree to which the cells have become differentiated overall, or both.

A fourth preferred embodiment of a method of inducing a terminally differentiated cell to proliferate further includes (d) providing a growth factor in the system; and (e) measuring the level of DNA synthesis in the system, an increase in the amount of the DNA synthesis, relative to the amount of DNA synthesis in a system lacking the candidate agent, indicating that the candidate agent is capable of inducing a terminally differentiated cell to proliferate. A "growth factor", as used herein, refers to a substance that promotes the growth of a cell, a tissue, or an organism, e.g., a vitamin, a hormone, or a protein or polypeptide, e.g., a member of the insulin-like growth factor family (IGF-I, II, or III), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), or fragments or analogs thereof.

A fifth preferred embodiment of a method of inducing a terminally differentiated cell to proliferate further includes: (d) providing a reporter gene in the system, the expression of the reporter gene being dependent on the interaction of the pocket protein with the factor; and (e) measuring the level of expression of the reporter gene, a decrease in the level, relative to the level of expression of the reporter gene in a system lacking the candidate agent, indicating that the candidate agent is capable of inducing a terminally differentiated cell to proliferate. The system can be, but is not of necessity, embodied by a transgenic non-human mammal.

The second aspect of the invention also includes a method for identifying an agent that mimics the effect of a factor required for terminal differentiation of a cell on a pocket protein. The method includes (a) providing a system, wherein the factor is absent or dysfunctional; (b) providing an agent in the system; and (c) observing

whether the agent mimics the effect of the factor on the pocket protein. As an embodiment, the agent can mimic the effect of the factor by binding to the pocket protein. The method can involve any combination of assays, e.g., DNA replication, cell growth, and/or DNA synthesis for measuring whether the agent mimics the effect caused by the interaction between a factor and a pocket protein. For example, where the system is a cultured cell line, and the observing involves measuring the level of DNA replication in cells of the cell line, a decrease in DNA replication, relative to the level of the DNA replication in cells not exposed to the candidate agent, indicates that the agent mimics the effect of the factor. Where the system is a cultured cell line and the pocket protein of the cells of the cultured cell line is absent or dysfunctional, the method can further include introducing an isolated DNA into the cells, the DNA being substantially homologous to a gene encoding a functional pocket protein. (This allows different species of pocket proteins to be individually introduced to the system, facilitating analysis of the interactions involving that particular species of pocket protein.) The level of cell growth of the cells exposed to the agent is then measured, a decrease in the level of cell growth relative to the level of cell growth of cells not exposed to the agent indicating that the agent mimics the effect of the transcription factors. By "cell growth" is meant an increase in the number of cells, or an increase in the density of cells. In another embodiment the method can further include measuring the amount of differentiation of the cells exposed to the agent, an increase in the amount of differentiation relative to the level of differentiation of cells not exposed to the agent indicating that the agent mimics the effect of the transcription factors.

A "cultured cell line" as used herein, refers to a homogeneous population of cells maintained under conditions suitable for the maintenance (where differentiated) and/or propagation (where undifferentiated) of the cell. By "the factor is absent or dysfunctional" is meant that the factor, or the gene for the factor, is missing from the system, or alternatively that the factor is present in a non-functional form, e.g., due to a mutation in the gene encoding the factor. "Mimics the effect of", as used herein, refers to the ability of the agent to simulate, or to closely imitate, the functional activity of the factor in its interaction with the pocket protein.

In a third aspect, the invention includes a method for detecting a mutation in a gene encoding a pocket protein. The method involves (a) providing a system that includes, e.g., by constitutive expression, a cellular factor required for terminal differentiation of a cell, the system being deficient in pocket protein function; (b) providing a candidate pocket protein that bears a mutation in the system; and (c) observing whether the pocket protein interacts with the factor, a decrease in the interaction, relative to the interaction of the factor with a wild-type pocket protein, indicating that the gene encoding the pocket protein bears the mutation. Also included within the scope of this aspect of the invention is a DNA molecule, fragment, or plasmid, the nucleic acid sequence of which encodes a pocket protein bearing the mutation detected by the above method. This DNA molecule is used in turn in a method of detecting, in a human fetus or patient, a genetic predisposition to develop an undesired proliferation of cells, e.g., a neoplasm. The method involves (a) providing the DNA fragment bearing the mutation in a pocket protein; (b) exposing the DNA fragment to a nucleic acid sample

obtained from the fetus or patient; and (c) detecting hybridization of the DNA fragment to the nucleic acid sample.

The invention further includes a method of identifying a factor that interacts with a pocket protein so as to induce terminal differentiation of a cell. The method involves either a) co-isolating, e.g., by immunoprecipitation, the pocket protein and the factor from the cell; b) providing a DNA expression library, e.g., a DNA phage expression library, specific for the cell-type of the cell, allowing the DNA of the library to be expressed, providing the pocket protein, and exposing the pocket protein to isolated samples of the DNA, e.g., phage plaques, binding of the pocket protein to the isolated sample or plaque indicating that the DNA of the isolated sample or plaque encodes the agent; or c) providing a complementation system that includes a selectable marker whose expression is under the control of a tissue-specific transcription factor, providing a pocket protein in the system, and observing the level of expression of the selectable marker, an increase in the level over background indicating that the system comprises the factor. A "DNA expression library" is a substantially complete sample of the DNA of a cell, e.g., the DNA of a brain cell, or of a muscle cell, inserted as DNA fragments into vectors, e.g., a phage vector, e.g., a lambda phage vector, which also include regulatory sequences that permit the expression of the genes encoded by the DNA. As used herein, a "complementation system" is a cell, e.g., a yeast cell, that is unable to produce a selectable marker. A "selectable marker" is a protein or nutrient that is either essential for the growth or survival of the cell, or one that, when expressed, produces a detectable quality, e.g., color, that allows

the cell to be easily distinguished from cells not expressing the selectable marker.

In a fourth aspect, the invention features a method of decreasing undesired cell proliferation. The method involves administering to the cell an agent that interacts with a pocket protein in the cell so as to mimic the effect between the pocket protein and a factor required for terminal differentiation of the cell. In preferred embodiments the agent may be a factor required for terminal differentiation of the cell itself, or a fragment or analog of a factor required for terminal differentiation. The agent may be any of the candidate agents listed above. The agent may interact with the pocket protein by binding, e.g., hydrogen binding, or covalent binding, to the pocket protein. The cell can be a neoplastic cell or a non-neoplastic cell. The cell can be extracorporeal or the cell can be in a mammal, e.g., a human. The pocket protein can be retinoblastoma protein, p107 protein, p300 protein, or any member of the pocket protein family. The agent can affect, e.g., induce, or enhance, the expression of a pocket protein. The factor is a "factor required for terminal differential of a cell" as defined above. For example, the factor is a member of the MyoD family of proteins, or a fragment or analog thereof. Alternatively, the factor is a member of the MEF2 family of proteins, or a fragment or analog thereof. The factor can also be a member of the MASH family of proteins, or a fragment or analog thereof. Preferably the agent maintains the active conformation of the pocket protein, e.g., by maintaining the active conformation by preventing phosphorylation of the pocket protein. The agent can be a nucleic acid, and can be administered, e.g., by a gene therapy technique. Where the cell is in a mammal, the method further comprises the administration of a transgene that enhances the

expression of a pocket protein in the mammal. The transgene can be administered to the mammal, or to an ancestor of the mammal, at an embryonic stage. Alternatively the transgene is administered to a somatic cell of the mammal. This method can be used to treat any type of undesired cell proliferation as defined above.

Another method of decreasing undesired cell proliferation involves administering to the cell an agent that induces the expression of a gene product in the cell so as to mimic the effect between the pocket protein and a factor required for terminal differentiation of the cell.

In a fifth aspect the invention features a method of inducing the replication of a cell in a terminally differentiated state. The method involves administering to the cell an agent that interacts with a pocket protein in the cell so as to decrease the interaction between the pocket protein and a factor required for terminal differentiation in the cell. The cell can be extracorporeal, or the cell is in a mammal. The cell can also be removed from a mammal, exposed to the agent, and returned to the mammal. The agent is a protein or a polypeptide, e.g., the peptide NLFCSEENDSSDD (SEQ ID NO:1), or a polypeptide substantially homologous to a portion of an oncoprotein, or an analog of a portion of an oncoprotein. The agent can bind to, e.g., by a hydrogen bond or a covalent bond, the domain of the pocket protein. This method can be used to regenerate a tissue in a mammal, e.g., a human. Preferably the method is used to regenerate a terminally differentiated tissue, e.g., muscle tissue, e.g., skeletal, cardiac or smooth muscle tissue; neural tissue; or epithelial tissue. The method is also used to induce the formation of lymphatic cells in a mammal, e.g., in a human.

"Homologous", as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., 5 positions in a polymer 10 subunits in length), of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC'5 and 3'TATGGC'5 share 50% homology.

An "isolated DNA", as used herein, refers to a nucleic acid sequence, segment, or fragment which has been purified from the sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, e.g., RNA, DNA, or proteins which naturally accompany it in the cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings are first described.

The drawings

Fig. 1 is a graphical illustration showing that SV40 T-antigen induces nuclear DNA synthesis, cdc2 Kinase activity and phosphorylation of pRB in terminally-differentiated C2 Muscle Cells.

Fig. 2 illustrates that MyoD arrests proliferation of pRB(+) but not of pRB(-) tumor cells.

Fig. 3 illustrates that MyoD induces myogenic differentiation in pRB(+) but not in pRB(-) Tumor Cells.

Fig. 4 illustrates that MyoD specifically binds to pRB *in vitro* and in muscle cells.

Fig. 5 illustrates that the C-terminal portion of the T/E1A-binding domain of pRB is sufficient for binding to MyoD.

Fig. 6 illustrates that the basic-helix-loop-helix domain of MyoD mediates interaction with pRB.

Fig. 7 illustrates the specificity of the MyoD-pRB interaction and as compared with bHLH E-proteins.

Fig. 8 illustrates interactions of pRB and E-box DNA-binding complexes *in vitro* and in cell extracts.

Fig. 9 illustrates the reentry of terminally differentiated myotubes into the cell cycle upon T antigen induction, by immunofluorescence staining, and by 5-bromodeoxyuridine (5-BrdU) incorporation.

Fig. 10 illustrates the myogenic factor sequences that interact with a pocket protein.

Fig. 11 illustrates the sequences of members of the MEF2 family that interact with a pocket protein.

Fig. 12 is an illustration of the relation between the amount of injected DNA and CAT-activity in a somatic tissue of a mammal.

Fig. 13 is an illustration comparing three different techniques for injection of a reporter gene construct into cardiac muscle.

Fig. 14 is an illustration of the time course of expression of injected gene constructs.

Fig. 15 is an illustration of the regional expression pattern of injected gene constructs throughout the left ventricular wall.

Fig. 16 is an illustration of the expression of promiscuous (MSV) or muscle-specific (-667rβ-MHC) promoter constructs in the right ventricle and in skeletal muscle.

Fig. 17 is an illustration of the correlation of CAT to luciferase activity in co-injection experiments.

Fig. 18 is an illustration of the mapping of the 5′ flanking region of the β-MHC gene *in vivo*.

The Invention

The experiments reported here document that a particular "active" configuration of the tumor suppressor retinoblastoma protein (pRB) is required for the production and maintenance of the terminally-differentiated phenotype of muscle cells. Applicants show that pRB inactivation, either through phosphorylation, binding to T-antigen, or genetic alteration, inhibits myogenesis. Moreover, inactivation of pRB in terminally-differentiated cells allows them to re-enter the cell cycle. In addition to its requirement for the myogenic activities of MyoD, pRB is also required for the cell growth-inhibitory activity of this myogenic factor. Applicants also show that pRB and MyoD directly bind to each other, both *in vivo* and *in vitro*, through the pocket and the basic-helix-loop-helix domains, respectively. Such binding maintains the active pRB configuration. All the results obtained are consistent with the proposal that the effects of MyoD on the cell cycle and the results of pRB on the myogenic pathway

result from the direct binding of the two molecules with each other.

EXAMPLE 1: SV40 T-antigen inhibits myogenesis and reverses the terminally-differentiated state of muscle cells

It occurred to applicants that pRB might be involved in the production of the terminally-differentiated state. To test this possibility, applicants used SV40 T-antigen as the test molecule. To this end, C2C12 myoblasts (hereafter referred to as C2; Yaffe et al., *Nature* 270:725-727, 1977; Blau et al., *Cell* 32:1171-1180, 1983) were permanently transfected with pMtSVTsNeo (Tegtmeyer et al., *J. Virol.* 8:516-524, 1971) carrying the neomycin resistance gene and encoding a mutant thermolabile T-antigen (Ts) controlled by the metallothionein promoter (Searle et al., *Mol. Cell. Biol.* 4:1221-1230, 1984). A second mutant of T-antigen, K1 (Kalderon et al., *Virology* 139:109-137, 1984; DeCaprio et al. 1988 supra; DeCaprio et al. 1989 supra; Kaelin et al., *Mol. Cell. Biol.* 10:3761-3769, 1990; Chen et al., *J. Virol.* 64:3350-3357, 1990), which neither binds to pRB nor transforms cells, was used as a control. Many neomycin resistant cell lines exhibiting similar properties were isolated after transfection with these plasmids. Two of these, C2Ts11, C2SVTts, and C2K1 were chosen for further analysis.

Applicants tested whether it was possible to induce re-entry into the cell cycle and reverse the terminally-differentiated state of myotubes by selective expression of T-antigen. To test this hypothesis, C2Ts11 and C2K1 myoblasts were induced to differentiate into myotubes at the non-permissive (40°C) temperature for the Ts T-antigen. Cultures that contained >95% of the nuclei in C2Ts11 and C2K1 myotubes were then stimulated by transfer to growth factor-rich medium at the permissive

temperature (33°C) and Ts or K1 T-antigen expression was induced by addition of 100 μM zinc.

Referring to Fig. 1a, it can be seen that DNA synthesis was re-induced in terminally-differentiated C2 myotubes expressing functional T-antigen. C2Ts11 and C2K1 myoblasts were fused to myotubes in differentiation medium (5% horse serum in DMEM) at the non-permissive temperature (40°C), then transferred to growth medium (20% serum in DMEM) containing 100μM Zn^{++} and 100μM BrdU for 22 hrs. at the permissive temperature (33°C). Myotubes were fixed and stained with monoclonal antibodies specific for T-antigen (upper panels) or BrdU to detect incorporation of BrdU into genomic DNA (lower panels), followed by a rhodamine-conjugated rabbit anti-mouse IgG (Sigma). The BrdU-positive nuclei in the C2K1 panel are myoblasts; no BrdU incorporation was detected in the C2K1 myotube nuclei.

The results of Fig. 1a show that when growth is stimulated in the presence of zinc, both C2Ts11 and C2K1 myotubes expressed high levels of T-antigen. As expected for terminally-differentiated cells, no DNA synthesis was detectable in the C2K1 myotubes, as determined by BrdU incorporation. The only cells in these cultures that stained positive for BrdU were residual undifferentiated myoblasts. In contrast, >95% of nuclei in C2Ts11 myotubes initiated DNA synthesis and eventually entered mitosis. This result demonstrates that induction of functional T-antigen is sufficient to re-induce DNA synthesis in terminally-differentiated cells.

The significance of this result is that cells that until now were believed to have been permanently withdrawn from the cell cycle can be induced to re-enter the S phase of the cell cycle. The failure of the K1 mutant to induce re-entry into the cell cycle suggests that the ability of T-antigen to interact with pRB is a

key feature of this effect. Thus, T-antigen expression not only inhibits myogenesis, but reverses the terminally-differentiated state when expressed in myotubes.

In Fig. 1b, cdc2-Kinase activity is re-induced in terminally-differentiated C2 myotubes expressing functional T-antigen. C2 or C2Ts11 cells were cultured in growth medium (GM) or differentiation medium (DM) at the non-permissive temperature, or transferred from DM to GM containing 100μM Zn^{++} for 22 hrs. at the permissive temperature (DM--GM*). Following immunoprecipitation of cell extracts by $p34^{cdc2}$ antiserum, cdc2-kinase activity was determined by *in vitro* phosphorylation of test substrate histone H1 using γ-[^{32}P]-ATP. Specificity of immunoprecipitation is shown by competition with the immunogenic peptide (C2Ts11, GM+pep).

Fig. 1b shows that $p34^{cdc2}$ kinase activity, measured by *in vitro* phosphorylation of the test substrate histone H1 is present in myoblasts (lanes labeled GM) and lost in wild-type and C2Ts11 myotubes (lanes labeled DM). In wild-type C2 myotubes, $p34^{cdc2}$ kinase activity, like pRB phosphorylation, is not re-induced in response to growth factor stimulation (lane labeled DM--GM). In contrast, C2Ts11 myotubes re-induce $p34^{cdc2}$ kinase activity (and pRB phosphorylation) when transferred to growth medium under conditions permissive for T-antigen expression and function (lane labeled DM--GM). Therefore, there is a direct correlation between $p34^{cdc2}$ kinase activity and pRB phosphorylation. This result provides further evidence that SV40 T-antigen induces terminally-differentiated myotubes to re-enter the cell cycle and suggests that $p34^{cdc2}$ kinase activity might be an effector of these changes.

pRB is re-phosphorylated in terminally-differentiated C2 myotubes expressing functional T-

antigen, as shown in Fig. 1c. Whole-cell extracts of metabolically-labeled C2 and C2Ts11 myotubes cultures as in (B), were immunoprecipitated with a pRB monoclonal antibody (XZ133) and analyzed by SDS-8%PAGE and autoradiography. pRB=unphosphorylated and ppRB=phosphorylated retinoblastoma protein.

T-antigen interacts only with the unphosphorylated form of pRB (Ludlow et al., *Cell* 56:57-65, 1989; Ludlow et al., *Cell* 60:387-396, 1990), which is the major form present in quiescent cells (Buchkovich et al. 1989 supra; Chen et al., *Cell* 58:1193-1198, 1989). Interestingly, as shown in Fig. 1c, there is direct correlation between the phosphorylated state of pRB in both parental C2 and the C2Ts11 myogenic cell derivatives, and the ability of the cells to re-enter the cell cycle. Both phosphorylated and unphosphorylated forms of pRB are present in cycling myoblasts of the two cell types (lanes labeled GM). Only the unphosphorylated form can be detected in differentiated myotubes (lanes labeled DM). In C2 myotubes, pRB remained unphosphorylated even after stimulation with 20% fetal calf serum (lane labeled DM--GM), in agreement with the failure of these cells to re-enter the cycle. This behavior is in contrast with that of C2Ts11 myotubes. In the presence of active T-antigen (33°C), these cells responded to growth factor stimulation by phosphorylating pRB, as it happens in non-terminally differentiated quiescent cells. Thus, pRB phosphorylation in response to growth factor stimulation of C2Ts11 myotubes parallels the ability of these cells to re-enter the cell cycle. This result provides additional evidence in support for the role of pRB in the production and maintenance of the terminally-differentiated state and suggested that the unphosphorylated form of this protein is the one involved in these functions.

EXAMPLE 2: pRB expression is required for the cell cycle arrest and the myogenic activities of MyoD

The results presented above offer compelling, but indirect, evidence that pRB plays an important role in muscle cell commitment, differentiation, and maintenance of the differentiated state. Forced expression of MyoD (and other members of the muscle-specific bHLH regulator family) induces many non-muscle cells into the myogenic pathway (Davis et al., *Cell* 51:987-1000, 1987; Weintraub et al., *Prod. Natl. Acad. Sci. USA* 86:5434-5438, 1989; Choi et al., *Proc. Natl. Acad. Sci. USA* 87:7988-7992, 1990; reviewed in Olson, 1990 supra, and Weintraub et al., *Science* 251:761-766, 1991). Moreover, MyoD expression has an important effect even in cells that are not converted to the myogenic pathway. In all cells tested, MyoD induced growth arrest independently of myogenesis, as demonstrated by the fact that mutations that abolish the myogenic activity of MyoD still are able to produce growth arrest (Sorrentino et al., *Nature* 345:813-815, 1990; Crescenzi et al., *Proc. Natl. Acad. Sci. USA* 87:8442-8446, 1990).

In order to directly investigate the role of pRB in the cell cycle arrest produced by MyoD, applicants decided to test the effects of forced expression of MyoD in non-muscle cells that are either wild-type or homozygous for a deletion that inactivates the RB gene product. Two human osteosarcoma cell lines, U2OS and Saos-2, have similar phenotypes but differ in their expression of pRB (Lee et al., *Science* 235:1395-1399, 1987a; Lee et al., *Nature* 329:642-645, 1987b; Huang et al., *Science* 242:1563-1566, 1988; Shew et al., *Cell Growth Diff.* 1:17-25, 1990a; Shew et al., *Proc. Natl. Acad. Sci. USA* 87:6-10, 1990b; Scheffner et al., *Proc. Natl. Acad. Sci. USA* 88:5523-5527, 1991). U2OS cells express wild-type pRB, whereas Saos-2 cells express a non-functional C-terminal deleted form. The growth

suppressive activity of MyoD was examined in these two cell lines by the colony forming efficiency (CFE) assay.

Parallel plates containing the same number of cells were infected with a virus encoding sense or anti-sense MyoD (Weintraub et al. 1989 supra), selected with neomycin and the number of colonies per plate scored fourteen days later. Fig. 2 shows representative plates (panel A) and colony counts (panel B) from this experiment.

In Fig. 2a, U2OS pRB(+) and Saos-2 pRB(-) osteogenic sarcoma cells were infected with retrovirus encoding either anti-sense or sense MyoD, plated at various cell densities, and selected for 14 days in neomycin, then parallel plates were stained with methylene blue and scored. Representative pairs of U2OS and Saos-2 plates (with different plating densities) are shown.

In Fig. 2b, calculation of percent reduction in colony forming efficiency (CFE) of a similar experiment to that shown in Fig. 2a. When infected with the sense MyoD virus, pRB(+) U2OS cells showed ~50% reduction in CFE as compared with the plate infected with the anti-sense MyoD virus. These results compared favorably with the previously reported effect of MyoD on cell growth in non-terminally differentiated cell types (Sorrentino et al. 1990 supra; Crescenzi et al. 1990 supra). In striking contrast, pRB(-) Saos-2 cells showed no growth suppression by the expression of MyoD, despite the fact that the level of expression of MyoD was similar in both cells lines. The lack of a growth-suppressive effect of MyoD in the absence of pRB was also observed in a second pRB(-) cell line, WERI-RB27 (Huang et al. 1988 supra). Applicants interpret these results to indicate that functional pRB is required for the MyoD-induced suppression of cell proliferation.

The differential effect of MyoD on cell growth in pRB(+) and pRB(-) cells, as shown above, prompted us to test the ability of MyoD to induce the myogenic pathway and muscle-specific gene expression in the same cell lines. pRB(+) U2OS cells and pRB(-) Saos-2 cells were plated and infected with the sense and anti-sense MyoD virus as in the previous experiment.

Referring to Fig. 3, U2OS pRB(+) and Saos-2 pRB(-) osteogenic sarcoma cells were infected with retrovirus encoding anti-sense or sense MyoD, selected in neomycin for 14 days, then transferred to DM for 3 days. Myogenic differentiation of U2OS but not Saos-2 cells is shown by cellular morphology in phase contrast images and by immunostaining for muscle-specific myosin heavy chain (MHC).

After selection in neomycin and transfer to differentiation medium, ~50% of the MyoD infected U2OS colonies converted to a myogenic phenotype, as determined by their cellular morphology and by immunostaining for expression of muscle-specific myosin heavy chain (Fig. 3, upper panels). As is the case for bona fide myogenic clones, there was a gradient of differentiation going from the center to the periphery of each clone. No conversion was detected in the U2OS cell plated infected with the anti-sense virus. In contrast, MyoD-infected Saos-2 colonies showed neither detectable morphological changes nor expression of myosin heavy chain (Fig. 3, bottom panels), independent of the length of culture in differentiation medium. This result provides evidence that, at least in the cell lines tested here, functional pRB is required not only for the MyoD-induced growth-arrest but also for its myogenic activity.

EXAMPLE 3: MyoD binds to pRB *in vitro* and in muscle cells

The results presented so far demonstrate a functional association between pRB and MyoD, two negative regulators of cell growth. Moreover, the effect of T-antigen and other pRB binding oncogenes on myogenesis presented here and reported by others (Webster et al., *Nature* 332:553-557, 1988; Endo and Nadal-Ginard 1989 supra; LaRocca et al., *Cell* 58:123-131, 1989; Miner and Wold, 1991) seems to be the opposite to that of MyoD.

To test whether pRB and MyoD have the capacity to physically interact with each other directly and specifically, a cellular extract from metabolically-labeled HeLa cells (see Methods) was incubated with bacterially-produced glutathione-S-transferase (GST) and a GST-MyoD fusion protein containing the region corresponding to amino acids 3-318 of MyoD (Lassar et al., *Cell* 58:823-831, 1989a).

Referring to Fig. 4a, GST-MyoD specifically binds the unphosphorylated form of pRB *in vitro*. [^{35}S]Methionine-labeled HeLa, or unlabeled HeLa, U2OS or Saos-2 cell extracts were incubated with GST or GST-MyoD protein beads. Total cellular proteins (lane labeled extract) and bound cellular proteins were separated by SDS-8%PAGE, then either directly autoradiographed (^{35}S-HeLa) or immunoblotted with a pRB monoclonal antibody. pRB=unphosphorylated and ppRB=phosphorylated retinoblastoma protein.

Fig. 4a shows that a protein with the mobility corresponding to pRB (105kD) was specifically bound to the GST-MyoD resin. To further confirm the identity of this protein, un-labeled cell extracts from proliferating HeLa, U2OS and Saos-2 cells, prepared in the presence of phosphatase inhibitors, were bound to GST or GST-MyoD proteins on sepharose beads. Bound proteins were eluted and analyzed by immunoblot with a pRB monoclonal antibody (see Methods). Both unphosphorylated and phosphorylated

forms of pRB were present in proliferating HeLa and U2OS cells, but, as expected, they were undetectable in the Saos-2 cells (extract lanes), which express a truncated and unstable form of pRB (Shew et al. 1990b supra). In HeLa and U2OS cells, GST-MyoD, but not GST alone, specifically bound the unphosphorylated form of pRB as identified by its electrophoretic mobility. In Saos-2 cells, no signal was detected. Thus MyoD directly and specifically binds to the unphosphorylated form of pRB *in vitro*.

To examine whether MyoD and pRB associate *in vivo*, applicants performed a double-immunoprecipitation assay using a cellular extract prepared from C2 myotubes. The first two lanes of Fig. 4b demonstrate the specificity of the MyoD antipeptide antiserum used for this experiment (see Methods). In Fig. 4b, MyoD is associated with pRB in differentiated muscle cells. [^{35}S]methionine-labeled C2 myotube extract was immunoprecipitated with a pRB monoclonal antibody (XZ133) using low-stringency buffer. Immunoprecipitates were disrupted by boiling in lysis buffer, then re-immunoprecipitated with pre-immune or MyoD antipeptide antiserum. The specificity of the MyoD antiserum was shown by immunoprecipitation of *in vitro* translated MyoD under the same conditions.

An extract from metabolically-labeled C2 myotubes was precipitated under low stringency conditions with a pRB monoclonal antibody. The anti-pRB precipitate was solubilized and re-precipitated under high stringency conditions with either pre-immune or anti-MyoD serum. The anti-MyoD serum specifically precipitated a C2 myotube protein with mobility identical to *in vitro* translated MyoD. No such molecule could be precipitated from BC3H1 cells, which do not express MyoD. These results indicate that MyoD is physically associated with pRB in terminally-differentiated muscle cells.

EXAMPLE 4: The bHLH domain of MyoD interacts with the pocket region of pRB and is competed by a T-antigen peptide

A sequence in pRB called the pocket domain, consisting of ~40% of the polypeptide, has been shown to be necessary for the binding of SV40 T-antigen and adenovirus E1A proteins *in vitro* (DeCaprio et al. 1988 supra; Whyte et al. 1988 supra; Kaelin et al. 1990 supra). Many of the point mutations affecting pRB function also map to this region of the molecule (Hu, Q. et al., *EMBO J.* 9:1147-1155, 1990), which has been shown to bind a number of cellular proteins, including E2F and c-myc (Kaelin et al., *Cell* 64:521-532, 1991; Bagchi et al., *Cell* 54:1063-1072, 1991; Chellappan et al., *Cell* 65:1053-1061, 1991; Chittenden et al., *Cell* 65:1073-1082, 1991; Rustgi et al., *Nature* 352:541-544, 1991). To determine whether MyoD also binds in the pocket region of pRB, labeled *in vitro* translated MyoD was tested for binding to a set of GST-pRB fusion proteins containing either the entire (amino acids 379-792) or the C-terminal portion of the pocket (amino acids 605-921) (Fig. 5a). [^{35}S]Methionine-labeled MyoD was incubated with GST or GST-pRB fusion protein beads as shown. The bound MyoD was analyzed by SDS-PAGE and autoradiography. Strong and efficient binding was detected with both proteins. A GST-pRB protein derived from a human tumor allele containing a point mutation within the pocket (C to F, amino acid 706) (Bookstein et al., *Science* 247:712-715, 1990; Horowitz et al., *Proc. Natl. Acad. Sci. USA* 87:2775-2779, 1990; Kaye et al., *Proc. Natl. Acad. Sci. USA* 87:6922-6926, 1990), which does not bind to T-antigen or E1A protein, bound MyoD was significantly lower efficiency than the wild-type sequence.

The reciprocal experiment was performed by binding C-truncated versions of *in vitro*-translated murine pRB to a GST-MyoD resin (Fig. 5b). *In vitro*-translated cyclin B

was used as a negative control for the specificity of binding. Various [^{35}S]methionine-labeled pRB proteins (see maps in C) were incubated with GST-MyoD and the bound proteins analyzed by SDS-PAGE autoradiography. Cyclin B is a negative control for non-specific binding of proteins to GST-MyoD. Fig. 5b shows that full-length murine pRB bound to GST-MyoD. A C-terminal deletion (m pRB/Sspl) containing amino acid residues 1-760 bound at very low efficiency, while a further deletion (m pRB/DraIII) containing amino acid residues 1-658, did not show detectable binding. A summary of the complete set of *in vitro* protein binding results is shown schematically in Fig. 5c. GST-pRB fusion proteins are human as in (Kaelin et al. 1991 supra) except for GST-pRB(605-921)*, which was constructed from murine pRB. These results mapped the MyoD binding domain of pRB to the region between residues 605 and 792, which includes the putative leucine zipper (Bernards et al., *Proc. Natl. Acad. Sci. USA* 86:6474-6478, 1989; Hu, Q. et al. 1990 supra). Single point mutations in this region that affect T-antigen and E1A binding to pRB also disrupt the binding of MyoD to pRB. Taken together, these results suggest that the binding site for T/E1A and related protein is overlapping but different from that of MyoD.

Interaction between MyoD and other HLH proteins (Murre et al., *Cell* 56:777-783, 1989), as well as with the leucine zipper protein c-Jun (Bengal et al., *Cell* 68:507-519, 1992), is mediated by the HLH domain of MyoD. To determine the domain involved in the binding to pRB, a panel of *in vitro* translated wild-type and internally-deleted MyoD proteins were tested for binding to a GST-pRB resin. A MyoD protein that contains a point mutation in the basic domain, which disables the myogenic activity of the protein, but not its effect on cell growth (B2proB3; Davis et al., *Cell* 60:733-746, 1990: Sorrentino

et al. 1990 supra) was also tested. The results obtained with these proteins are shown in Fig. 6a and schematized in Fig. 6b.

Similar amounts of various [^{35}S]methionine-labeled MyoD proteins (see maps in B) were incubated with GST-pRB. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography.

Fig. 6 shows that the bHLH domain is required for binding of MyoD to GST-pRB. Alteration of the basic domain, by substituting the alanine at position 114 for a proline, did not affect MyoD binding in this assay, which is consistent with the ability of this mutant to inhibit cell growth in a biological assay (Sorrentino et al. 1990 supra). However, when the entire basic domain was deleted from MyoD, no binding to pRB was observed. In addition, the HLH protein Id, which lacks a basic domain, failed to bind pRB in the same type of assay. Finally, the bHLH domain of E2-2 effectively bound *in vitro* translated pRB.

Although pRB bound to all bHLH proteins thus far tested, MyoD had the highest affinity for pRB, as demonstrated by comparison of the pRB binding of *in vitro* translated bHLH E-proteins, E2-2 and E12, the usual heterodimerization partners of MyoD (Murre et al., *Mol. Cell Biol.* 11:1156-1160, 1991; Lassar et al., *Cell* 66:305-315, 1991). Fig. 7b shows how GST-pRB binds to MyoD with higher affinity than to other bHLH proteins. [^{35}S]Methionine-labeled E2-2, E12, or MyoD proteins were incubated with GST-pRB(379-928) and the bound proteins analyzed by SDS-PAGE and autoradiography. Lysate lanes show that similar amounts of each protein were loaded on the GST-MyoD beads. Exposure time was 12 hrs. The two E-proteins, although able to bind pRB, do so at an efficiency that is at least an order of magnitude lower then MyoD. Together, these data suggest that the basic

and HLH regions are both necessary and sufficient for binding to pRB.

To test whether T-antigen and MyoD bind to pRB in a competitive manner, GST-MyoD was bound to labeled pRB in the presence of different concentrations of wild-type or mutant T-antigen peptides. Fig. 7a shows that binding of pRB to GST-MyoD is completed by wild-type but not by mutant T-antigen peptide. [^{35}S]Methionine-labeled murine pRB was incubated with GST-MyoD in the presence of equal amount wild-type (T) or mutant (K1) peptide. Bound proteins were analyzed by SDS-PAGE and autoradiography. The wild-type T-antigen peptide containing amino acids 102-114 (T peptide), which has been shown to bind pRB (Kaelin et al. 1990 supra), very effectively competed with pRB binding to MyoD, whereas an equivalent peptide, containing a single substitution that abolishes pRB binding (K1 peptide) (DeCaprio et al. 1988 supra; 1989 supra; Chen and Paucha 1990 supra), had no detectable effect on the MyoD-pRB interaction (Fig. 7a). Therefore, T-antigen and MyoD compete for the binding to pRB. Interestingly, MyoD does not contain the highly conserved pRB-binding motif (LXCXE; X=any amino acid (SEQ ID NO:2)) that is essential for pRB-binding of large T, E1A and E7 proteins, as well as two cloned cellular proteins for pRB binding (Defeo-Jones et al., *Nature* 352:251-254, 1991).

From this set of results, applicants conclude that MyoD interacts with pRB in a specific manner that is competed by T-antigen and that involves the bHLH and pocket domains, respectively, of these two molecules.

EXAMPLE 5: Association of pRB with the E-box-binding activity of muscle cell extracts

In muscle cells, the transcriptionally active E-box binding complex contains a heterodimer of MyoD and E-protein (Lassar et al. 1991 supra). Since pRB binds to

both MyoD and E2-2 proteins, it was of interest to determine whether this interaction occurred with MyoD-Ed-2 heterodimers. To examine this question and, at the same time to determine whether binding to the E-box affected the interaction of these proteins with pRB, gel-shift assays were performed using a probe containing three copies of the muscle creatine kinase enhancer E-box and various GST fusion proteins (see Methods). The GST fusion proteins as shown were incubated with a [^{32}P]-labeled DNA probe containing three copies of the muscle creatine kinase E-box (MCK probe) and analyzed by gel shift assay. Free probe ran off the gel. Homodimers of either MyoD or E2-2, as well as MyoD-E2-2 heterodimers, all bound to this DNA element, but neither pRB nor GST bound (Fig. 8a). When GST-pRB was added to MyoD alone, before addition of the DNA binding site, its binding to DNA was completely disrupted, demonstrating that the pRB-MyoD heterodimer does not bind to the E-box DNA *in vitro* (Fig. 8a). Since MyoD homodimers efficiently bound to this probe, the inhibition of binding by pRB suggested that MyoD has a higher affinity for pRB than for itself. In contrast, E2-2 homodimers and MyoD-E2-2 heterodimers were not disrupted by addition of pRB (Fig. 8a). In fact, pRB appeared to chase the E2-2 homodimers (faster migrating complexes) into MyoD-E2-2 heterodimers (slower migrating complexes). pRB may stabilize the E-box binding bHLH protein heterodimer.

In vivo, pRB is found in multi-protein complexes containing the transcription factor E2F (Bagchi et al. 1991 supra; Chittenden et al. 1991 supra). However, the pRB-E2F complex cannot be reconstituted *in vitro* with purified proteins, because additional nuclear factors are required (Bandara et al., *Nature* 352:249-251, 1991; Hiebert et al., *Genes and Dev.* 6:177-185, 1992). For this reason, gel-shift assays were performed with muscle

cell extracts to ascertain whether pRB is associated with the E-box binding complex in muscle cells. The anti-pRB monoclonal antibody, XZ133 (Hu, Q. et al., *Mol. Cell. Biol.* 11:5792-5799, 1991), was used for these experiments. In Fig. 8b, pRB monoclonal antibody diminishes E-box binding activity in muscle cell extracts. GST-MyoD+GST-E2-2 or whole cell extracts from C2 myotubes or RD cells were incubated with PAB101 (control T-antigen monoclonal Ab), XZ133 (pRB monoclonal antibody), 100-fold molar excess E-box or unrelated DNA competitor, or GST-pRB, and analyzed by gel shift using the MCK probe as in (A). The complex specifically diminished by pRB antibody in C2 myotubes is marked by an arrow. The left hand panel of Fig. 8b shows that neither this antibody nor a negative control monoclonal antibody (PAB101) affected the binding of bacterially-produced MyoD+E2-2 proteins to the triple E-box probe. In contrast, the XZ133 anti-pRB antibody specifically diminished E-box binding activity of both C2 mouse muscle cell and human embryonal rhabdomyosarcoma tumor cell (RD: Hiti et al., *Mol. Cell. Biol.* 9:4722-4730, 1989; Wellington et al., *Proc. Natl. Acad. Sci. USA* 89:1755-1759, 1992) extracts. Note that in Fig. 8b and 8c, due to the GST moiety, the fusion proteins generated a different migration pattern than did the endogenous complexes. Addition of GST-pRB fusion protein to either extract did not affect the E-box binding activity or pattern. These results suggest that pRB is a component of the E-box binding complex in muscle cells.

To obtain further evidence for the presence of pRB in the E-box binding complexes in muscle cells, applicants exploited the competition for pRB binding between MyoD and T-antigen and related proteins. Shirodkar et al. (*Cell* 68:157-166, 1992) have recently shown that pRB- and p107-containing E2F DNA-binding

complexes in cell extracts can be disrupted by addition of GST-E1A fusion protein (GST-E1A 976). A truncated version of this protein (GST-E1A 922) that binds poorly to pRB and p107 has little effect on these complexes. These two E1A fusion proteins were used to test their effect on the E-box binding complexes present in a C2 myotube extract. In Fig. 8c, pRB-binding protein E1A diminishes E-box binding activity in muscle cell extract. GST-MyoD+GST-E2-2 or a C2 myotube extract were incubated with GST, wild-type GST-E1A 976 or mutant GST-E1A 922 and analyzed by gel shift with the MCK probe. Free probe ran off the gel in the C2 myotube lanes. Fig. 8c provides evidence that neither GST, GST-E1A 976, nor GST-E1A 922 had any effect on the gel-shift pattern of bacterially-produced MyoD+E2-2 heterodimers, demonstrating that E1A does not interact with these two proteins. In contrast, E1A 976 protein specifically and efficiently disrupted the E-box binding activity present in C2 myotube extracts. The slight diminution of binding observed with the truncated form of E1A is consistent with previous observations (Shirodkar et al. 1992 supra). These results strengthen those obtained with the pRB antibody, and together strongly support the contention that pRB is present in the E-box binding complex from muscle cells. The disruption of E-box binding activity by E1A correlates with its inhibitory effect on myogenesis and muscle-specific gene expression (Webster et al. 1988 supra). This observation provides a rationale for the requirement of pRB or related proteins for the myogenic activity of MyoD, and for the inhibition of this process by T-antigen.

EXAMPLE 6: Reentry of terminally differentiated myotubes into the cell cycle upon T antigen induction.

Fig. 9 illustrates the reentry of terminally differentiated myotubes into the cell cycle upon T

antigen induction. (A). Mature T antigen-transfected myotube cultures were shifted to GM containing Zn^{2+} (and BrdU in panels a-d) and incubated at 33°C (to allow T antigen expression) for 27 hr (panels a-e) or 34 hr (panel f) before fixing and immunofluorescence staining. To examine G1/S progression induced by T antigen expression, cells were co-stained for T antigen induction with an anti-T mAb and with an anti-BrdU mAb to monitor DNA synthesis (respectively panels a and b, same cells are shown) and for the presence of cyclin A with affinity-purified anti-cyclin A antibodies and DNA replication again with anti-BrdU mAb (respectively panels c and d, same cells are shown). Hoechst 33258 staining of DNA was used to analyze progression through G2/M phase. Shown in panel e are prophase nuclei with condensed chromosomes (higher magnification of the same field is depicted in the inset). Panel f shows a multinucleated myotube (nuclei are visualized with Hoechst dye) undergoing cytokinesis. Microtube forming the midbody visible in the cleavage furrow were stained with an anti-a tubulin mAb. (B). Control C2 myotubes treated as T antigen-transfected ones were assayed for DNA synthesis and cyclin A presence by double staining with anti-BrdU mAb (panel b) and anti-cyclin A antibodies (panels c and d). Shown are phase (panel a) and immunofluorescence (panels b-d) micrographs of the same field. Panel c represents a 6x- overexposure of panel d to better display the absence of cyclin nuclear staining emphasized by the cytoplasmic background level. Arrows in panel b point to replicating unfused C2 myoblasts.

To correlate induction of DNA synthesis with the expression of large T antigen, C2SVTts and C2 untransfected myoblasts were allowed to fuse into multinucleated myotubes and then shifted to GM/Zn containing the thymidine analog 5-bromodeoxyuridine

(BrdU) at 33°C for one day before fixing. Large T antigen expression was assayed by indirect immunofluorescence with a mouse monoclonal antibody that recognizes only the large T protein, while DNA synthesis was monitored by indirect immunofluorescence with an anti-BrdU mAb. C2SVTts but not untransfected C2 myotube nuclei showed BrdU staining in most of T antigen positive nuclei (Fig. 9 A[a,b] and B[a,b], respectively). Contrary to myotube nuclei, unfused myoblasts when shifted to GM, show BrdU incorporation (arrows in Fig. 9 B[b]). Cyclin A synthesis was then correlated to BrdU incorporation by indirect immunofluorescence using an anti-human CA polyclonal antiserum (Girard et al., *Cell* 67:1169, 1991). As described earlier for the T antigen immunofluorescence, most C2SVTts myotube nuclei GM/33/Zn2+ stained positive for CA while almost no signal was detected in control C2 myotube nuclei (Fig. 9 A[c] and B[a,d], respectively; an overexposure of the same field of Fig. 9 B[a,d] is shown in Fig. 9 B[c] for a better visualization of the negative cyclin A staining). DNA replication, as detected by BrdU incorporation, was observed in most cyclin A positive nuclei, though not in all (Fig. 9 A[c,d]). Reverse cases, where BrdU positive nuclei stained negative for cyclin A, were also observed (arrows in Fig. 9 B[b,d]). In some cases, strong cyclin A labelling did not correlate with BrdU staining (arrow in Fig. 9 A[c,d]). The general pattern of cyclin A staining observed varied from a nuclear punctate disperse pattern to a more intense nuclear signal excluded from the nucleoli. The immunofluorescence pattern was independent of the fixation method and was similar using a different anti-cyclin A polyclonal antiserum. In some myotube nuclei, progression through G2/M phase was detected upon T antigen induction. Condensed chromosomes (Fig. 9 A[e]) as well as metaphase and anaphase figures

were detected and these were accompanied by nuclear envelop breakdown and dispersion of the T antigen throughout the cytoplasm. Also in some cases multinucleated myotubes were viewed undergoing cytokinesis (Fig. 9 A[f]).

EXAMPLE 7: p107

p107 is present in the E2F transcriptional factor multi-protein complex. The constituents of this complex, including p107, vary during the phases of the cell cycle. As yet, the p107 gene product has not qualified as a bona fide tumor suppressor because it is not located at a site of malignancy-associated deletion in the human genome, and, despite intensive search, no tumor cells with functionally defective p107 proteins have been identified. In both *in vivo* and *in vitro* assays, p107 can function much like pRB as a suppressor of cellular growth.

Applicants have shown that in cultured muscle cells, p107 is abundantly expressed in the proliferating myoblasts, but p107 expression is extinguished in terminally-differentiated myotubes. In fact, the expression of the p107 and pRB genes is reciprocally regulated during myogenesis in cultured cells. The p107/pRb myogenic switch, which was observed at both the mRNA and protein levels, suggests that p107 is the major pocket protein required during myoblast proliferation, while pRb plays a more important role in the differentiation process. The role of p107 is concentration-dependent, however, since p107, when present at high concentrations, mimics pRB. This is explained more fully as follows.

The fact that mouse cells with targeted disruption of both pRb alleles (pRb-/pRb-) are fully competent to undergo myogenic factor-induced cell cycle arrest and

terminal differentiation initially presented a paradox, as the data presented herein imply an essential role for pRb in these processes. However, when the Rb-/Rb- myoblasts were examined for expression of p107, they were found to aberrantly up-regulate p107 with muscle cell differentiation. This remarkable result suggests that the Rb-/Rb- myoblasts, which were originally derived from embryonic stem cell teratomas grown in SCID mice, exhibit compensatory over-expression of the p107 gene, and implies that, in the total absence of pRb, p107 can functionally substitute for pRb in the cell cycle arrest and myogenic functions *in vivo*.

Indeed, our binding data demonstrated that, like pRb, p107 binds with high specificity and high affinity to the myogenic factors. This association was easily demonstrated by *in vitro* binding assays, but was also found in human rhabdomyosarcoma cells *in vivo*. The so called "pocket domain" of p107 is structurally similar to that of pRb, except for a segment called the "spacer." The spacer of p107 binds to cyclin A but the equivalent segment of pRb does not bind to cyclin A. Thus cyclin A can discriminate between pRb and p107. Applicants have now shown that MyoD (in the form of a GST fusion protein) can also be used to discriminate between p107 and pRb. Whereas *in vitro* translated p107 and pRb both bind to GST-MyoD with equivalent apparent affinity, the binding of pRb to GST-MyoD can be competed with peptides containing the pRB-binding sequences of either SV40 large T antigen or the E2F-1 transcriptional factor. Very high concentrations of these peptides failed to perturb the interaction of p107 and GST-MyoD. Thus, the binding of MyoD to pRb or p107 can be distinguished by differential competition with these two peptides. Furthermore, binding experiments also show that p107 cannot simultaneously bind to cyclin A and MyoD. This contrasts

with the transcriptional factor E2F, which can be identified in a multi-component complex containing both p107 and cyclin A proteins, among others.

In addition to these binding experiments, applicants have also shown that p107 has functional properties similar to Rb. Mouse myoblasts that lack pRb but express p107 retained their susceptibility to cellular transformation and suppression of myogenic differentiation by SV40 large T-antigen. Presumably, SV40 T-antigen blocks myogenesis in these cells by inhibiting the interaction between myogenic factors and p107. In the Saos-2 myogenesis assay, when co-transfected with a myogenic factor such as myogenin, p107 functions like pRb to induce growth arrest and activate the myogenic pathway. It is important to note that Saos-2 cells have endogenous p107 protein that appears to be functional but insufficient to induce myogenesis. As in the mouse Rb-/Rb- myoblasts, it appears that over-expression of the p107 molecule (which is normally down-regulated at the onset of myogenic differentiation) is necessary for this process. Additionally, applicants have shown that over-expression of human p107 in mouse C2C12 myoblasts drives these cells out of the cell cycle and prematurely activates myogenesis. Importantly, this occurs in the presence of growth factors, which suggests that the cell cycle arrest and myogenic signals delivered to the myoblast by constitutively-expressed p107 are dominant over the growth-promoting and differentiation-suppressing signals of growth factors. This result has important implications for the ultimate use of these molecules in anti-proliferative therapy.

Thus, although p107 is normally associated with growing myoblasts, it appears to functionally substitute for Rb as a collaborator of myogenic factors in the growth arrest and differentiation of muscle cells.

Compensatory over-expression of the p107 gene may explain the lack of a muscle phenotype in the mouse embryos that lack pRb.

EXAMPLE 8: Methods

A: Plasmids

Plasmids encoding GST-pRB(379-792), GST-pRB(379-928) and GST-pRB(379-928;706C-F) were prepared as described in Kaelin et al. (1991 supra, hereby incorporated by reference). The plasmid encoding GST-MyoD(3-318) was prepared as described in Lassar et al. (1989a supra).

The plasmid encoding GST-pRB(605-921) was generated as follows: The plasmid pJΩpRB (Bernards et al. 1989 supra), which contains the full length mouse pRB cDNA, was digested with BgIII (which cleaves in the sequences at the codon for amino acid 605 and at the 3′ untranslated sequence). The BgIII fragment of pRB was then cloned into the BamH1 site of the GST2 vector. This resulted in a plasmid expressing a fusion protein of the GST protein followed by amino acids 605-921 of the mouse pRB. To generate a plasmid for the *in vitro* transcription and translation of pRB, pJΩpRB was first linearized by digestion with BamH1 and then subjected to a partial digestion with BgIII. This 2.8 kb BamH1-BgIII fragment encoding the entire pRB sequence was cloned into the BamH1 site of psp73 (Stratagene). This yielded a plasmid containing the full length mouse RB cDNA coupled to bacterial phage promoter T7.

The plasmid pMtSVTtsNeo contains cDNA encoding the thermolabile SV40 large T-antigen coupled to the mouse metallothionein promoter and also contains the neomycin resistance gene linked to the herpes simplex virus -1 thymidine kinase promoter. pmtSVneo is based on the vector pKP45, which was made by deleting nt. 676-2365

from pBR322. It consists for 2.5 kb HindIII-BamH1 fragment of SV40ts A58 mutant (Tegtmeyer et al. 1971 supra) gene, which encodes thermolabile Large T and wild type small t, fused to mouse Mt-I gene promoter (1.8 Kb). At the Sal1 site the plasmid also contains the neomycin resistance gene linked to herpes simplex virus-1 thymidine kinase gene promoter as a selection marker. pMtNeo was constructed by deleting HindIII-BamH1 sequence coding for T antigens from pMTSVTtsNeo.

The plasmid pMtK1Neo was generated by replacing the HindIII-BamH1 fragment of the T-antigen with the DNA fragment encoding the mutant form of the large T (K1).

B: Cell Culture, Transfection, Infection, and Immunofluorescence

The mouse skeletal muscle cell line C2C12 was maintained in growth medium (20% FCS in Dulbecco's Modified Eagle's Medium (DMEM)) at 37°C. Cells were transfected using the calcium-phosphate co-precipitation method (Graham and Van der Eb, Virology 52:456, 1973 hereby incorporated by reference). After 10-12 days, G418-resistant (500μg/ml) colonies were isolated and amplified. The transfected clones were maintained in growth medium at 37°C. Among the neo-resistant clones was sued the one that show a high level of Large T protein at 33°C together with a high percentage of fusion at 40°C in DM). THe expression of Large T in this clone was verified by norther blot and by immunofluorescence. C2Ts11 and C2K1 cells were allowed to form mature myotubes for five days at 40°C in differentiation medium.

Untransfected mouse skeletal muscle cell line C2C12 was maintained in Growth Medium (20% Fetal Calf Serum in DMEM) at 37°C. Terminal differentiation was induced where necessary by changing cells 50% confluent from GM to 5% Horse Serum in DMEM (Differentiation Medium-DM). Transfection of C2C12 cells with pMtSVTtsNeo

was obtained following the calcium phosphate coprecipitation medium. The myotubes were then transferred to growth medium containing 100 μM zinc at 33°C. Whenever myotubes, either transfected or untransfected, were used for experiments requiring the absence of myocytes that could spontaneously reenter the cell cycle, cells were treated with 9 ug/ml of cytosinarabinoside (Sigma, St. Louis, MO) for 48 hr and then kept for 24 hr in DM; cells used for following experiments consisted of >95% myotubes.

C: [3H]TdR Labelling and Autoradiography

Cell cultures on 35-mm plastic dishes were incubated with 0.3 μC/ml {6-3H}TdR (specific activity, 20.0 μC/mmol; Amersham Corp.) in appropriate media and processed for autoradiography as described (Endo and Nadal-Ginard 1986 supra). Specimens were recorded by phase-contrast microscopy. [3H]TdR incorporation rate was measured by routine methods (*Ausubel et al.* supra).

U2OS (A.T.C.C. #HTB96), Saos-2 (A.T.C.C. #HTB85), WERI-RB27 (A.T.C.C. #HTB169) and RD (A.T.C.C. #CCC136) cells were purchased from A.T.C.C. and maintained in DMEM containing 10% FCS. Viral infection was performed as described in Weintraub et al. (1989 supra; hereby incorporated by reference).

D: Immunofluorescence

The immunofluorescence analysis was performed as follows: Cells were fixed by direct immersion in 3.7% paraformaldehyde in phosphate buffered saline PBS for 10 minutes. Fixed cells were then permeabilized by immersion in 0.3% TX100 in PBS for 5 minutes, washed three times with PBS, and incubated with specific antibodies. Primary antibodies used for immunofluorescence were PAB101 (monoclonal large T-antigen antibody; A.T.C.C. #TIB117), MF20 (monoclonal myosin heavy chain antibody), and monoclonal BrdU

antibody (Boehringer). The secondary antibody was rhodamine-conjugated rabbit anti-mouse IGg (Sigma, St. Louis, MO). Cells were examined by immunofluorescent microscopy using a Zeiss 40X objective lens. DNA synthesis was measured by BrdU incorporation as described in Sorrentino et al. (1990 supra; hereby incorporated by reference).

For the experiments shown in Fig. 9, cells were seeded onto gelatin-coated glass coverslips at a density of 3x103 cells per cm2 and allowed to attach in mitogen-rich medium (DMEM plus 20% fetal calf serum, GM). Myogenic differentiation was induced by changing the cells to DMEM containing 5% horse serum (DM) and incubating at 37°C for 4-5 days. Expression of T antigen in these fully differentiated myotubes was achieved by shifting the cells to GM plus Zn2+ (0.1 mM) and incubating at 33°C. DNA synthesis was monitored by adding the thymidine analog BrdU (0.1 mM, Sigma) to the Zn2+-containing GM. Cells were washed once with PBS, fixed for 10 min in 3.7% formalin in PBS, quenched for 10 min in 50 mM glycine and permeabilized with 0.25% Triton X-100 in PBS for 10 min. After blocking 10 min in 3% BSA in PBS, cells were stained for 60 min with the primary antibodies: immunoaffinity-purified rabbit anti-cyclin A antibody diluted 1:100 (Girard et al., 1991 supra), anti-T antigen mAb (Pab101, American Type Culture Collection), anti-a tubulin mAb (kindly provided by Dr. T. Arai, University of Tsukuba, Japan). After extensive washes with 0.1% NP-40 in PBS, cells were incubated 60 min with biotinylated donkey anti-rabbit immunoglobulins (Amersham, dilution 1:200), biotinylated goat anti-mouse IgG (Amersham, dilution 1:200) and fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories), respectively. The biotinylated antibodies were followed by incubation for 30 min with streptavidin-Texas red (Amersham,

dilution 1:500) and washed thoroughly with 0.1% NP-40 in PBS. All dilutions were made in 3% BSA in PBS and all incubations were carried out at room temperature except for the anti-cyclin Ab incubation, which was at 37°C. For co-staining with BrdU, cells were fixed again in formalin, quenched in glycine and incubated 10 min in 1.5 M hydrochloric acid followed by 10 min washes with PBS and finally stained for 60 min with fluorescein-conjugated anti-BrdU mAb (Boehringer Mannheim, 50 mg/ml). For Hoechst staining, cells were incubated with 1 mg/ml Hoechst 33258 (Boehringer Mannheim) in PBS for 1 min. After washing in 0.1% NP-40 in PBS coverslips were mounted in 90% glycerol in PBS. Specimens were examined and photographed on a Nikon Microphot-SA microscope equipped with phase-contrast and epifluorescence optics, using 40x and 100x planapochromat lens. Pictures were recorded on Kodak Ektar 100 film.

E: Immunoprecipitation

Double immunoprecipitation assay was performed as described in Lassar et al. (1991 supra) with a few modifications. The first immunoprecipitation was performed by immunoprecipitation of metabolically labeled C2 myotubes in low stringency buffer (EBC buffer: 50mM Tris pH8, 120mM NaCl, 0.5% NP40, 100mM NaF, 200μM Na orthovanadate) containing 10μg of protease inhibitors aprotinin (Sigma, St. Louis, MO), PMSF (Sigma, St. Louis, MO), and leupeptin (Boehringer) using pRB monoclonal antibody XZ133 (Hu, Q. et al. 1991 supra). The precipitate was washed four times in NETN buffer (20mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40), resuspended in 50μL of SDS-lysis buffer (20mM Tris pH7.5, 50mM NaCl, 0.5% SDS and 1mM DTT) and heated to 95°C for two minutes. The supernatant was removed and diluted with 500μL NETN buffer together with either preimmune or immune MyoD antisera and protein-A beads. MyoD antiserum

was made against a peptide (KVEILRNAIRYIEGL)(SEQ ID NO:4) from the second helix region of murine MyoD and showed no apparent cross-reactivity with *in vitro* translated E12 or E2-2. The precipitated proteins were washed four times with NETN buffer, boiled in SDS buffer, and analyzed by SDS-10%PAGE and autoradiography.

In other experiments, cells were metabolically labelled for 3 hr with 0.2 mCi/ml of trans[35S]-label (NEN) in 3 ml of DMEM methionine-free. Cell dishes were washed in ice-cold PBS, scraped and collected in Eppendhorf tubes. After a brief spin in the microfuge, cells were lysated by adding 1ml of lysis buffer (50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 0.1% NP-50, 5 mM EDTA, 50 mM NaF) supplemented with 1 ug/ml aprotinin (Sigma, St. Louis, MO), 1 ug/ml Leupeptine (Boehringer), 75 ug/ml PMSF (Boehringer). After having homogeneously suspended the cells in the lysis buffer, tubes were rocked at 4°C for 1 hr; cell extracts were then centrifuged at 13000 rpm and the supernatants were transferred into new tubes; aliquots were taken fro assessment of radioactivity by liquid scintillation counting of the acid-precipitable cpm. Samples were precleared with 40 ug of a 50% (v/v) protein A- Agarose suspension (Repligen, Cambridge, MA), rotating for 1 hr at 4°C. The total counts/min/ml were normalized prior to adding the appropriate antibody. Proteins were immunoprecipitated by rotation at 4°C for 1 hr followed by the addition of protein A-sepharose beads (40 ul 50% v/v) and another rotation for 1 hr at 4°C. In the case of anti-pRB antibodies, 20 ug of anti-mouse IgG Garose (Sigma, St. Louis, MO), was added instead of protein A Agarose. Immune complexes were purified by three washes of beads with 0.5 of lysis buffer. Samples were suspended in 50 ul of Laemmli buffer, boiled and analyzed by SDS-PAGE. The anti-RB monoclonal antibody XZ-133 was a generous gift of Drs. Harlow and Hu (Hu, Q.

et al. 1991 supra). Anti-cyclin A antiserum was a gift of Dr. J. Pines (J. Pines et al., *Nature* 346:760, 1990). The western blot for p34 cdc2 was done by using an anti-C terminus antibody (Gibco, Gaithersburg, MD), following the manufacturer's instructions.

F: cdc2-Kinase Activity Assay

Protein kinase assays were performed as described by Giordano et al. (*Cell* 58:981-990, 1989, hereby incorporated by reference). Briefly, cell lysates, normalized for protein content by Bradford assay (Biorad, Hercules, CA), were immunoprecipitated with 2μg of polyclonal $p34^{cdc2}$ antibody (Gibco, Gaithersburg, MD). Immunoprecipitates were incubated for 5 minutes at 30°C in a 50μL reaction mix containing 50mM Tris (pH7.4), 10mM $MgCl_2$, 1 mM DTT, 50μg/ml histone H1 and 5μCig-[^{32}P]-ATP. Reactions were stopped with 2X Laemmli buffer and analyzed by SDS-12%PAGE and autoradiography.

G: *In vitro* Binding Assay

Expression and purification of Glutathione S-transferase fusion proteins were performed as described in Kaelin et al. (1991 supra). Expression and purification of Glutathione S-transferase fusion proteins were performed as the following. Cultures of Escherichia coli transformed with GST recombinants were diluted 1:10 in Luria-Bertani medium (LB) containing ampicillin (200 g/ml) and incubated for 3-5 hr at 37°C with shaking. After 1 hr of growth, isopropyl-B D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. Fusion proteins were recovered by centrifugation at 5000xg for 10 min at 4°C and resuspended in 1/10 vol of PBS. The bacteria were then lysed on ice by sonication and centrifuged at 10,000xg for 10 min at 4°C. Bacterial supernatant were rocked for 30 min at 4°C with glutathione-Sepharose. The glutathione-Sepharose beads were then washed four times with PBC. Various *in vitro* translated proteins (10μL) were incubated with different GST fusion protein beads (20μg fusion protein with 25μL of glutathione-sepharose) for 2 hr at 40°C. Beads were then washed five times with NETN buffer. Bound proteins were released by boiling in SDS loading buffer and separated on SDS-PAGE.

H: *In vitro* Competition Assay

The *in vitro* competition assay was performed as described above but in the presence of 75μg of T-antigen (NLFCSEEMDSSDD) (SEQ ID NO:1) amino acids NLFCSKEMDSSDD (SEQ ID NO:3)) or K1 (amino acids 102-114 of large T,107 Glu-Lys) peptides. Western blotting was as described by Rustgi et al. (1991 supra), using monoclonal antibody against pRB (Pharmingen G3-245).

I: DNA-Binding Assays

GST, GST-MyoD, GST-E2-2 (containing only the bHLH domain of human E2-2), GST-m pRB (605-921), GST-E1A 976 and GST-E1A 922 (Shirodkar et al. 1992 supra) proteins were grown and purified as described (Kaelin et al. 1991 supra). Approximately 100 ng of each fusion protein was combined in 15μL binding reactions containing 1μg double-stranded poly (dIdC) in 20 mM HEPES (pH 7.6), 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, with 10-20 φmole of double-stranded oligonucleotide containing three tandem copies of the muscle creatine kinase high-affinity E-box (upper strand: 5′-CGGATCCAAcacctgctgcctaacacctgctgcctaac acctgctgcctaagcttg-3′) (SEQ ID NO:5) labeled with Klenow DNA polymerase and α-[^{32}P]-dATP. C2C12 myotube and RD whole cell extracts were prepared as described (Pagano et al., *Science* 255:1144, 1992; hereby incorporated by reference); 2μL of each extract were used for binding reactions. Extracts were incubated with 3μL of hybridoma supernatant or 100 ng GST fusion proteins for 20 min. at room temp. followed by addition of probe and further incubation at room temp. for 20 min. DNA-protein complexes were analyzed by 5%PAGE in 1X TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA; pH8) and autoradiography.

J: Colony Forming Efficiency (CFE) assay

CFE assay was performed as described in Crescenzi et al. (1990 supra) hereby incorporated by reference). Briefly, U20S and Saos cells were infected with virus encoding either sense or anti-sense MyoD (Weintraub et al. 1989 supra). G418 (800μg/ml) selection began two days after infection, and dishes were stained with methylene blue 14 days later. The number of colonies in MyoD-infected dishes were compared to that in anti-sense MyoD-infected control dishes.

EXAMPLE 9: Administration of genetic material to a somatic tissue of a mammal

A reporter gene whose regulatory sequences are under the control of the interaction between a pocket protein and a cellular factor is injected into the somatic tissue of a non-human mammal. As an example, applicants have tested the following protocol for injecting the DNA constructs into the cardiac tissue of a dog. Analogous DNA constructs and other mammalian species can be easily substituted.

A: Plasmids

MSV-CAT was created by fusing the coding sequence of the chloramphenicol acetyl transferase (CAT) gene (Gorman et al. 1982. *Mol.Cell.Biol.* 2:1044-1051) to the long terminal repeat of the mouse sarcoma virus (MSV). RSV-Luciferase was described previously (DeWet et al. 1987. *Mol.Cell.Biol.* 7:725-737). The series of deletions of the 5′ flanking region of the β-MHC included the -3300rβ-MHC-CAT, -667rβ-MHC-CAT, -354rβ-MHC-CAT and -215rβ-MHC constructs, which are genomic fragments of the rat β-MHC gene from -3300 base pairs (b.p.),-667 b.p., -354b.p., and -215 b.p. to +38 b.p. relative to the transcriptional start site cloned in front of the CAT gene (Thompson et al. 1992. *J.Biol.Chem.* 266(33):22678-22688). -607rα-MHC-CAT contains position -607 to +32 of the rat α-myosin heavy chain promoter sequence linked to the CAT gene (Widom et al. 1991. *Mol.Cell.Biol.* 11:677-687). -256ApoAI-CAT contains nucleotide sequence -256 to +500 of the 5′ flanking sequence of the rat apolipoprotein AI fused to the CAT gene (Widom et al. *Mol. Cell. Biol.* 1991, 11:677-687).

B: Animals

Adult mongrel dogs of either sex weighing between 20 and 26 kg were used for these experiments. Dogs were premedicated with xylazine (10mg/kg i.m.) and general anesthesia was induced with thiamylal (10-20mg/kg i.v.) and maintained with halothane (0.5-1.5 vol.%). Observing

sterile technique, the pericardium was opened and exposed through a lateral thoracotomy at the 5th intercostal space. The pericardium was opened and the heart was anchored with a suture through the apex. Up to thirty 4mm^2 patches of Dacron were sewn to the epicardium to mark injection sites. Following placement of the patches, circular plasmid DNA resuspended in 1 x phosphate buffered saline was injected through a 30 ga needle inserted perpendicular to the epicardium. The incision was closed in layers and the chest was evacuated. The animals were observed during recovery until fully conscious. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the "Guide for the Care and Use of Laboratory Animals" (Dep. Health and Human Svcs, NIH publication No. 86-23).

C: Tissue Preparation

After seven days the animals were killed with an overdose of pentobarbital and the heart was rapidly excised and placed in ice-cold saline. The labeled injection sites were excised as transmural blocks of myocardium, weighing 0.5 to 1.0 g, and immediately placed in liquid nitrogen. Tissue was stored at -80°C until further processing. Immediately prior to the CAT-assay, tissue was homogenized in 1 ml of homogenization buffer containing 25mM glycyl-glycin (pH 7.8), 15mM $MgSO_4$, 4mM EGTA (pH 8.0), 1mM DTT as described previously (Kitsis et al. *Proc. Natl. Acad. Sci., USA* 1991, 88:4138-4142). The suspension was centrifuged at 6000g for 15 minutes at 4°C and the supernatant was used for further analysis. The supernatant was normalized for protein content as determined by the Bredford-Assay (Bio-Rad) by the appropriate dilution with homogenization buffer.

D: Cat-Assays

Cat-assays were performed as previously described (Seed et al. *Gene* 1988, 67:271-277). In brief, 10% of the supernatant normalized for protein content, 1 μl ^{14}C-labeled chloramphenicol (0.25mCi) and 5μl of n-buturyl Coenzyme A (5mg/ml) were mixed and filled to a total volume of 125μl with 250 mM Tris-HCl, pH 8.0. The reaction mixture was incubated at 37°C for 2 hours, which was in the linear range of the reaction. The acetylated chloramphenicol fraction of the suspension was extracted by adding 300μl of xylene. Suspensions were back extracted twice with 250mM Tris-HCl, pH 8.0. Aliquots of 200μl were counted in scintillation fluid in a β-counter (Beckman LS 6000IC, Beckman Instruments).

E: Luciferase-Assay

Luciferase-assays were performed as described elsewhere (Brasier et al. *BioTechniques* 1989, 7(10):1116-1122). In summary, 10% of the supernatant normalized for protein content was brought to a volume of 100μl with homogenization buffer (see Tissue Preparation) and mixed with 360μl of reaction buffer (25mM glycyl-glycin (pH 7.8), 15mM $MgSO_4$, 4mM EGTA (pH 8.0), 1mM DTT, 15mM KPO_4 pH 7.8, 2mM ATP, 0.3% Triton X-100). Light emission was measured in a monolight luminometer (1251 Luminometer LKE Wallac, Turqu, Finland) immediately after the addition of 0.2mM D-luciferin to the reaction mixture. Light units are expressed as the integral of activity measured over 20 seconds. Only values within the linear range were included for analysis.

F: Data Analysis

All data are reported mean ±standard error of the mean (SEM). For statistical comparisons of CAT-activity across time and regionally within the left ventricle, ANOVA was used. ANOVA was also employed for promoter comparisons and comparison of injection techniques. When significant, inter-group comparisons were performed with

unpaired t-tests with the Bonferroni adjustment. Linear regression analysis was used to examine the correlation between CAT- and luciferase-activities in the co-transfection experiments. All analyses were performed on a Macintosh computer using Statview II (Abacus Concepts Inc., Berkeley, CA) with a $P<0.05$ considered significant

G: The expression of gene constructs injected intothe myocardium follows dose-response kinetics.

In order to determine the efficiency and kinetics of expression of DNA injected into canine myocardium applicants injected a constant volume of 200µl containing increasing amounts of MSV-CAT plasmid DNA ranging from 10 to 300µg per injection site into one dog heart.

Fig. 12 shows a dose-response relation between the amount of injected DNA and CAT-activity on a scatter plot of CAT-activity (in counts per minute/100) per injection site, versus the total amount of DNA (MSV-CAT) per injection site. (Means (±SEM) are shown as solid squares, n=4 for each dose. Linear regression function is shown ($P<0.001$).) An amount as little as 10µg of DNA resulted in a CAT-signal nearly 10 times above background. In the range from 10µg to 200µg the dose-CAT-activity relationship appeared linear ($y = 0.2x + 10.8$; $r^2 = 0.54$). Higher amounts of total DNA resulted in a plateau reflecting a saturation kinetic of DNA uptake, transcription or both. These results indicate that the canine myocardium has a large capacity for uptake of injected DNA over a very broad range of concentrations. However, the slope of the curve clearly indicates that the efficiency of expression is the highest at the lower concentrations. The reasons for this behavior are not known at this time. However, it stresses the requirement for internal standards when the efficiency of expression between different constructs and/or amounts of injected DNA are to be compared.

H: Amount of injury imposed on injection site does not correlate with the level of gene expression

Three different injection techniques were compared in one experiment to analyze the impact of injection induced injury on the cardiac tissue and its relevance for the level of expression of the injected reporter gene constructs. Because it could be argued, that the results presented here represent uptake by non-myocyte cells and therefore are not meaningful for the study of cardiac muscle biology, applicants tested a reporter construct, whose expression is restricted to muscle cells. Although, to date there has been no published report identifying the pattern of myosin heavy chain expression in the canine myocardium, our RNA blot analysis indicates, that, like in other large mammals, the normal adult isoform is β-MHC, with low level of α-MHC. For this reason, applicants chose to use previously characterized β-MHC promoter constructs for this analysis (Thompson et al. *J. Biol. Chem.* 1992, 266(33):22678-88). In the first group, 200μl of -667rβ-MHC-CAT plasmid DNA solution were injected via one single injection. In the second group, 50μl of the same concentration of DNA were injected 4 times per injection site. To account for differences in the distribution of the DNA solution in the tissue between those two groups a third group was included, where 200μl of the DNA-solution, containing the same amount of total DNA as the other two groups, were injected via one injection and three additional stabs with the needle (but without injection of DNA) were performed around the actual injection site.

Fig. 13 shows a comparison of three different injection techniques. A total amount of 50 μg of -667 rβ-MHC-CAT was injected in each group. (Means (±SEM) n=10 for each group.) There were no statistical differences

in CAT-activity between any of these groups (Anova, $p>0.05$). However, because of the apparent trend of higher expression in the 4 times 50µl group and because of a lower standard deviation in this group, applicants used this injection technique for subsequent experiments.

I: Promiscuous and tissue-specific reporter gene constructs are expressed over extended periods of time.

To evaluate the stability and peak of the expression of injected recombinant gene constructs in canine myocardium applicants sacrificed animals at 4 different time points (day 3, 7, 14, and 21 post injection). Multiple injections of CAT gene constructs utilizing either promiscuous (MSV) or muscle specific (-667rβ-MHC) promoters were performed. Fig. 14 shows a time course of expression of injected gene constructs. CAT-activity (in counts per minute/1000 versus days post injection for promiscuous (MSV, solid bars) and muscle specific (-667rβ-MHC, hatched bars) promoters regulate expression of the CAT reporter gene. (Mean ± SEM, n=5 for each time point (*P<0.01 compared with day 7)).

Analysis of variance (ANOVA) for CAT-activity within the MSV and -667rβ-MHC promoters was significant ($P<0.01$, for the MSV and -667rβ-MHC constructs). The overall temporal pattern of expression of the exogenous genes was similar between promiscuous and tissue-specific promoter constructs with CAT-activity well detectable already 3 days p.i., a peak at day 7 and a subsequent decline in CAT-activity throughout day 21 (MSV is $P<0.01$ and -667rβ-MHC is $P<0.0001$, unpaired-t test). This pattern of expression at the protein level is likely to be an overestimate of duration of expression of the injected DNA due to the long half life of the CAT-protein, which is over 50 hours in most cell types (Thompson et al. *Gene* 1991, 103:171-177). Therefore, the levels of expression shown in Fig. 13 not only reflect

the activity of the driving promoter, but also reflect phenomena beyond the transcriptional level, e.g. half-life of the expressed protein as well as that of the injected episomal DNA.

J: Reporter gene constructs are evenly expressed throughout the left ventricular wall

Since it was the purpose of our study to establish a practical model to compare the regulation of different recombinant gene constructs within the same animal by injecting at numerous sites into the canine myocardium, applicants compared the expression of a given construct at different locations throughout the myocardium. To account for regional differences in uptake and/or expression of foreign DNA by the cardiocytes, applicants injected the muscle-specific construct -667rβ-MHC-CAT in 24 different sites of the left ventricle. Fig. 15 shows the regional expression pattern of injected gene constructs throughout the left ventricular wall that was obtained, with 4 columns around the left ventricle each comprising 6 injection sites ranging from base to apex (see cartoon). Means ± SEM of each column are shown on the left hand panel 9 (n=6). Means ± SEM of each row are shown on the right hand panel (n=4).

There were no detectable regional differences in CAT-expression (ANOVA, $p<0.05$); however, because of the apparent trend toward decreased expression at the extreme base and apex of the left ventricle, applicants elected not to inject at those locations for subsequent experiments.

K: CAT-activity is about 3-fold higher in the left than in the right ventricle

One of the advantages of the canine versus the rodent model is the possibility to perform multiple injections also into the right ventricle.

Fig. 16 shows an expression of promiscuous (MSV) or muscle-specific (-667rβ-MHC) promoter constructs in the right ventricle and in skeletal muscle. Values (mean ± SEM) are depicted as percent of expression of the same construct in the left ventricle (= 100%, solid bars). Open bar is right ventricle (n=10 for MSV, n=8 for -667rβ-MHC). Hatched bar is skeletal muscle (n=10 for MSV, n=9 for -667rβ-MHC. The expression of promiscuous as well as tissue-specific promoter constructs was found to be a third to that observed in the left ventricle. This phenomenon could be based on the difference in wall thickness with a smaller number of cells being transfected along the injection tract in the right ventricle. Alternatively, this may reflect a higher chance of leakage of injected DNA into the myocardial cavity during injections into the right ventricle.

L: <u>The heart expresses injected reporter gene constructs 1 to 2 orders of magnitudes higher than the skeletal muscle.</u>

In order to compare the level of expression of injected gene constructs in the canine heart with other organs, and possibly detect organ related differences in expression of exogenous genes, applicants performed injections of the promiscuous MSV-CAT and the muscle-specific β-MHC-CAT constructs also into the quadriceps, a skeletal muscle of mixed fiber types. Values in Fig. 16 are expressed as percent of the expression of the according construct in the left ventricle. The expression of both, the muscle-specific and promiscuous promoter constructs, was respectively about one and two orders of magnitude lower in the skeletal muscle than in the left ventricle. As demonstrated before on mRNA level in the rat, the β-MHC is most abundant in the soleus, a skeletal slow twitch muscle, and in hypothyroid animals also in the cardiac ventricle (Izumo et al. *Science* 1986, 231:597-600). Thus, the low level of expression of the

β-MHC promoter construct may be due to the fact, that applicants injected the DNA into a mixed fiber muscle, where the β-MHC protein is much less abundant than in slow-twitch fiber muscle. The reason for the lower expression of the promiscuous construct in the skeletal muscle compared to the heart is unknown.

M: Co-injection of a control gene construct (RSV-luciferase) has proven useful to monitor transfection efficiency in this model

Co-transfection is used to control for transfection efficiency *in vitro* and it has also been used *in vivo* (Kitsis et al. *Proc. Natl. Acad. Sci., USA* 1991, 88:4138-4142; Parmacek et al. *Mol. Cell Biol.* 1992, 12:1967-1976), but its usefulness in *in vivo* experiments has not been evaluated before. This is particularly important, since in contrast to cell culture studies the transfected cell pool consists of a heterogeneous cell population, which may express the two gene constructs in a diverse pattern rendering the co-injection useless. Two representative experiments were analyzed for correlation of CAT- versus luciferase-expression in this model.

Fig. 17 shows the correlation of CAT-to Luciferase-activity in co-injection experiments. Scatter plot of CAT-activity (counts per minute) versus luciferase-activity (light units). 100 μg of a tissue-specific (-667rβ-MHC-CAT, Fig. 5a) or promiscuous (MSV, Fig. 5B) reporter gene construct were co-injected with 20 μg of a control gene construct (RSV=Luciferase). The regression functions are as indicated. In one experiment the muscle specific -667rβ-MHC-CAT construct was co-injected with the RSV-luciferase gene (Fig. 17, A). The long terminal repeat of the rous sarcoma virus (RSV-LTR) functions as a relative promiscuous promoter as determined previously, where transgenes directed by the RSV-LTR were highly expressed in tissue of mesodermal

origin (Overbeck et al. *Science* 1986, 231:1574-1577; Swain et al. *Cell* 1987, 50:719-727). The correlation between CAT- and luciferase-activity was significant (r^2=0.8, slope=0.8±0.2, P<0.01). When two promiscuous promoter constructs (MSV-CAT, RSV-Luciferase) were co-injected (Fig. 17, B) the linear regression analysis of CAT-activity vs. luciferase-activity revealed an r^2 of 0.9, with a slop of 3.5 ±0.6, P<0.005 (n=6). This indicates that co-transfection is meaningful and necessary to account for transfection efficiency *in vivo*, and therefore differences in expression of gene-constructs may be attributed to differences in regulation of expression of these constructs as long as values are normalized for the activity of the co-transfected gene. This analysis also reveals the feasibility of co-injecting a tissue-specific promoter construct with a promiscuous promoter construct to control for transfection efficiency.

N: Injection of reporter gene constructs into canine myocardium as a useful method to detect regulatory gene sequences *in vivo*

After characterization of many important parameters relevant for the regulated expression of injected reporter gene constructs, applicants addressed the questions of the feasibility of this model for mapping promoter sequences and thus identifying regulatory gene sequences *in vivo*. Constructs using serial deletions of the 5′ flanking region of the β-MHC gene cloned in front of the CAT reporter gene were used (Fig. 18).

Fig. 18 shows the mapping of the 5′ flanking region of the β-MHC gene *in vivo*. A series of deletions of the upstream region of the rat β-MHC gene ranging from -3300 to -215 relative to the transcription start site were cloned in front of the CAT gene and injected into the canine myocardium. For comparison -607rα-MHC-CAT and

-256 ApoAi-CAT were also injected. 100 μg of reporter gene construct were co-injected with 20 μg of a control gene construct (RSV-Luciferase). CAT-activity was corrected for luciferase-activity and is expressed in percent of MSV-CAT. Open bars are β-MHC-CAT constructs (n=6-10). The hatched bar is the α-MHC-CAT construct (n=10). The solid bar is the Apo AI-CAT construct (n=10).

As a negative control applicants used a construct containing the nucleotide sequence -256 to +397 relative to the transcription start site of the apolipoprotein AI gene, which has been shown to be expressed in hepatocytes specifically (Widom et al. *Mol. Cell Biol.* 1991, 11:677-687). Activity of the different β-MHC constructs was compared with ANOVA ($p=0.001$). All six possible pairwise comparisons were made and found to be significant ($p<0.005$), except -354rβ-MHC versus -215rβ-MHC. The most active construct was the -667rβ-MHC-CAT reporter gene construct. The marked difference in activity compared to the other β-MHC-CAT constructs agrees with the presence of a positive regulatory element between position -667 and -354 relative to the transcription start site. Further deletion of the β-MHC gene promoter to position -186 relative to the transcription start site (-186rβ-MHC-CAT) resulted in a sharp decline of the CAT-activity to a level, which was barely above that of the negative control construct (-256 ApoAI-CAT), indicating another positive regulatory element important for basal transcription between position -215 and -186 relative to the transcription start site. A repressor element may be located further upstream as implied by the drop of activity of the -3300rβ-MHC-CAT construct to about a fourth of the activity of the -354 and -215rβ-MHC-CAT constructs. The -607rα-MHC-CAT construct, which is the most active tissue-specific construct in rat cardiocytes

as demonstrated by transfection assays of primary cell cultures (Mahdavi et al. *Cellular and Molecular Biology of Muscle Development*, Alan R. Liss Inc. 1989, pp. 369-379), was about 6-fold less active than the -667rβ-MHC-CAT. This result was expected, given the relative level of endogenous β- and α-MHC mRNA expressed in large mammals versus small mammals.

Taken together, our observations demonstrate, that direct DNA injection into the canine myocardium is a practical and efficient method to study gene regulation in the intact animal.

EXAMPLE 10: Techniques useful in the methods of the Invention

The methods of the invention can be conducted by using the following techniques, as well as by using the methods provided herein, and methods routinely known to those skilled in the art. (All of the following citations are hereby incorporated by reference.)

A. A System. A "system" can be: 1) An *in vitro* system, e.g., an *in vitro* system based on a buffer, e.g., phosphate buffered saline or Tris buffer, into which the purified components are added. An example of an *in vitro* system for measuring the interaction between two proteins, as well as an *in vitro* system for measuring the interaction of a protein with a DNA recognition sequence, e.g., a DNA enhancer sequence, is described herein. 2) An *in vitro* "system" can also be based on a cellular extract; e.g., the cellular extract described herein. 3) An *in vivo* system is based on either a cell line, examples of which are provided throughout the text, or an animal model. Additional examples of differentiated cell lines include a neural cell line (A.T.C.C. #PC12), a smooth muscle cell line (Rothman et al., *Circulation* 86:1977-1986, 1992) Taubman et al., *Circulation Res* 70:314-325, 1992), or an epithelial cell line such as a

keratinocyte cell line (Green et al., *Cell* 11:405-415, 1977). Saos-2 cells are pRB^- osteosarcoma cells and U2OS cells are pRB^+ osteosarcoma cells (described herein). Cell lines that are constitutive for the expression of a protein of interest, e.g., a transcription factor, can be selected by methods known to one skilled in the art. For example, to provide a system, e.g., a cell, in which to test the functionality of pocket protein alleles, it is necessary to select for constitutive expression of a transcription factor, e.g., MyoD or myogenin. This is done by introducing into a cell line, e.g., a pRB^- cell line, e.g., Saos-2, a DNA construct in which a drug resistance gene, e.g., the neomycin resistance gene, is functionally linked to regulatory sequences recognized by the transcription facter, e.g., the E-box sequence, which is recognized by MyoD and Mash factors, or the MEF2 consensus enhancer sequence (Yu et al., 1992 supra). Neomycin resistant clones are selected. These manipulations are routine and known to one skilled in the art (Ausubel et al. supra; Sambrook et al. supra).

An *in vivo* system can also be embodied by a transgenic non-human animal. Methods of providing a transgenic animal which bears a transgene in a somatic tissue are provided as part of the present invention. Transgenic animals bearing a germ-line transgene are prepared by pronuclei injection using standard protocols as described by Hogan, B., Constantini, F., and Lacy, E. (1986) Manipulating the Mouse Embryo: A Lab. Manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY). These protocols, with the necessary modifications, are used to produce transgenic animals useful for screening agents that are in turn useful for decreasing or increasing cellular proliferation.

Where the assay involves a reporter gene, the transgenic animals are made using complete reporter gene

coding sequences. The expression of the reporter gene sequences can be targeted to different tissues and stages of development through the use of tissue- and developmentally-specific promoters and/or enhancers. Preferably these promoters and/or enhancers are specifically recognized by the particular transcription factor and tissue-type of interest.

The certainty that the transgene will be expressed in the transgenic mammal is illustrated by the ability of a MEF2 construct to be expressed in a whole animal, as has been demonstrated by direct injection of the DNA constructs into skeletal and cardiac muscle in the present invention, using a modification of the direct DNA injection described by Wolff (1991 *Biotechniques* 11:474-485). Using this methodology applicants have shown that it is possible to produce high level expression in cardiac and skeletal muscle of the injected MEF2, lasting for at least 30 days after injection.

B: Nucleic Acid Sequences: Nucleic acid coding sequences useful for producing protein components for the assay described herein include: pRB (Bernards et al., *Proc. Natl. Acad. Sci. USA* 86:6474-6478, 1989; Lee et al., *Science* 235:1395-1399, 1987), p107 (Ewen et al., 1991 *supra*), MyoD and myogenin (Wright et al., *Cell* 56:607-617, 1989), MEF2 (Yu et al., 1992 *supra*), MASH factors (Johnson et al., 1992 *supra*; Johnson et al. 1990 *supra*), and RSRF factors (Pollack et al., 1991 *supra*), as well as the sequences of the oncoproteins SV40 T antigen, adenovirus E1A, and polyoma virus Py protein, and papilloma virus E7 protein, the sequences of which are known to the art. The protein components are produced by either transfecting a cultured cell line with the gene to produce the protein *in situ*, or by expressing the gene and purifying the protein. For instance, polypeptides useful as components in a method of the invention, or as

candidate agents in a method of the invention, can be purified using conventional methods of protein isolation known to one schooled in the art, e.g., methods including but not limited to precipitation, chromatography, immunoadsorption, or affinity techniques (see, e.g., Scopes, R. Protein Purification: Principles and Practice, 1982 Springer Verlag, NY). The polypeptide can be purified from starting material using the genomic DNA, or from a recombinant form of the cDNA genetically engineered into an overproducing cell line. Where the component or agent is a polypeptide fragment of one of the proteins encoded by the above genes, the polypeptide fragment can by synthesized by automated peptide synthesis (Ausubel et al. eds. Current Protocols in Molecular Biology, John Wiley & Sons, publ. NY. 1987, 1989; Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press).

Genes encoding the pocket proteins and transcription factors defined in this invention can also be used as agents for the methods described herein, i.e., can be added to one of the assays described herein, or can be administered to a mammal as a therapeutic, e.g., by gene therapy techniques or by transgenics. In addition, the regulatory regions upstream of these genes in the 5′ direction are useful for directing the expression of a reporter gene.

C: Enhancers recognized by tissue-specific transcription factors are useful to the methods and DNA constructs provided by the invention. For instance, the MyoD and Mash families of transcription factors, as well as other basic-helix-loop-helix transcription factors, recognize the E-box consensus nucleic acid sequence CANNTG ((Lassar et al. 1991 supra). The MEF2 and RSRF family recognize the MEF2 enhancer sequence (Pollack et al., Genes Dev. 5:2327-2341; 1991; Yu, et al. 1992

supra). The enhancer upstream of the β-myosin heavy chain gene (β-MHC) is also recognized by a muscle-specific transcription factor(s) (Thompson et al. *J. Biol. Chem.* 266:22678-22688, 1991). These enhancer sequences are provided as examples and are not to be construed as limiting the invention. Other tissue-specific enhancers are provided for by the art.

D: Reporter Genes: The method of measuring the level of reporter gene product is appropriate to the reporter gene product used on the recombinant DNA molecule in the NGF promoter assay. For example, where the reporter gene encodes an enzyme, an enzymatic assay is used. The concentration of reporter gene product is determined by providing excess substrate for that enzyme, under conditions in which the enzyme concentration is the limiting factor on the reaction, and measuring the concentration of product produced. In one example, the CAT gene (Promega, Madison, WI) encodes the enzyme chloramphenicol acetyl transferase, the level of which can be determined by providing acetyl CoA and chloramphenicol and measuring the production of acetylated chloramphenicol. When luciferase is used as the reporter gene (Promega, Madison, WI), the cells hosting the recombinant DNA molecule are lysed, adenosine triphosphate (ATP) and luciferin are added to the lysate in a liminometer, and the resulting light emission is correlated with the amount of luciferase present in the lysate (Nardeen et al., *Biotechnique* 6:451 (1988)). Beta-galactosidase (An et al., *Mol. Cell. Bio.* 2:1628 (1982)) (Promega, Madison, WI) is measured with chromogenic substrates, i.e., colorless substrates which are hydrolyzed to yield colored products, e.g., o-nitrophenyl-β-D-galactoside (Promega, Madison, WI), which are then measured spectrophotometrically or by the formation of colored bacterial colonies. More detailed

descriptions of reporter gene assays are available in the art (Ausubel et al. *supra* 1989; Miller et al. Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1972. pp. 352-355). Other reporter genes and assays for their corresponding protein products are equally suitable and known to those skilled in the art.

E: Co-isolation of Pocket Proteins and Factors or Agents: Methods of co-isolating a pocket protein and a cellular factor are provided herein. For example, a pocket protein can be identified by co-immunoprecipitation using an anti-factor antibody, e.g., an anti-MyoD antibody. A cellular factor that interacts with a pocket protein can be identified by co-immunoprecipitation, using an antibody specific for a pocket protein, e.g., an anti-pRB antibody, as demonstrated herein. Methods of preparing additional polyclonal or monoclonal antibodies are routine to those skilled in the art (Hu et al., *Mol. Cell. Biol.* 11:5792-5799, 1991; Ausubel et al. supra).

F: Cellular Assays and Growth Factors: Cellular assays for measuring the ability of the pocket protein/transcription factor interaction to influence cell proliferation are demonstrated herein, including methods of assaying for cellular differentiation, e.g., by immunofluorescence; methods of assaying DNA replication, i.e., DNA synthesis, e.g., by BrdU incorporation; or methods of assaying for cell growth. Growth factors useful for determining the ability of a cell to respond to a growth factor include but are not limited to IGF-I, II, or III, PDGF, or FGF, as defined and referenced herein.

G: Modes of Administration of a Therapeutic Composition:

The agents of the invention can be administered to a mammal, particularly a human, by any appropriate method suitable for the particular agent, e.g., orally, parenterally, transdermally, or transmucosally. Administration can be in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery using micelles, gels or liposomes. Therapeutic doses are determined specifically for the agent at hand, most administered within the range of 0.001 - 100.0 mg/kg body weight, or within a range that is clinically determined as appropriate by those skilled in the art.

The nucleic acids of the invention can be administered to a mammal, preferably a human, or a domesticated animal, by techniques of gene therapy. An appropriate recombinant vector, e.g., an attentuated virus, is administered to a patient in a pharmaceutically-acceptable buffer (e.g., physiological saline). The therapeutic preparation is administered in accordance with the condition to be treated. For example, retroviral vectors, can be used as a gene transfer delivery system for a nucleic acid agent. Numerous vectors useful for this purpose have been described (Miller, 1990 *Human Gene Therapy* 1:5-14; Friedman, 1989 *Science* 244:1275-1281); Eglitis et al. 1988 *Biotechniques* 6:608-614; Tolstoshev et al. 1990 *Current Opinion in Biotechnology* 1:55-61; Sharp, 1991 *The Lancet* 337:1277-1278; Cornetta et al., 1987 *Nucleic Acid Research and Molecular Biology* 36:311-322; Anderson 1984 *Science* 226:401-409; Moen, 1991 *Blood Cells* 17:407-416; and Miller et al. 1989 *Biotechniques* 7:980-990). Retroviral vectors are particularly well developed and have been used in a clinical setting (Rosenberg et al. 1990 *N. Engl. J. Med.* 323:370).

Where it is necessary for the nucleic acid, or a polypeptide agent encoded by the vector, to enter the nucleus, it may be necessary to employ an attenuated viral vector that naturally replicates and is expressed in the nucleus. Alternatively, the nucleic acid vector can include a nuclear localization region, e.g., two consensus regions consisting of basic amino acids separated approximately by 10 "spacer" amino acids. This region is likely to be responsible for directing the transport of this protein from the cytoplasm, where it is produced, to the cellular nucleus (Dingwall, C. and Laskey, R., 1991. *Trends in Biochemical Sciences*. 16:478-481).

The retroviral constructs, packaging cell lines and delivery systems which may be useful for this purpose include, but are not limited to, one, or a combination of, the following: Moloney murine leukemia viral vector types; self inactivating vectors; double copy vectors; selection marker vectors; and suicide mechanism vectors.

H: Non viral methods for the therapeutic delivery of nucleic acid encoding an agent of the invention

Nucleic acid encoding an agent of the invention under the regulation of a tissue-specific promoter, and including the appropriate sequences required for autonomous replication or for insertion into genomic DNA of the patient, may be administered to the patient using the following gene transfer techniques: microinjection (Wolff et al., Science 247:1465 (1990)); calcium phosphate transfer (Graham and Van der Eb, Virology 52:456 (1973); Wigler et al., Cell 14:725 (1978); Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413 (1987)); lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413 (1987); Ono et al., Neuroscience Lett 117:259 (1990); Brigham et al., Am. J. Med. Sci. 298:278 (1989);

Staubinger and Papahadjopoulos, Meth. Enz. 101:512 (1983)); asialorosonucoid-polylysine conjugation (Wu and Wu, J. Biol. Chem. 263:14621 (1988); Wu et al., J. Biol. Chem. 264:16985 (1989)); and electroporation (Neumnn et al., EMBO J. 7:841 (1980)).

I: Miscellaneous Methods: Methods of preparing a DNA library or a DNA expression library, e.g., a phage cDNA expression library, e.g., λgr10, or λgt11, and for screening a DNA library or expression library, are known to those skilled in the art. The library can be screened with a labelled nucleic acid probe, e.g., a fragment of a gene encoding a pocket protein, a transcription factor, an oncoprotein, or a kinase or phosphorylase, or the expression library can be screened with a labelled antibody. These methods are routine and known to the art (Sambrook et al. supra; Ausubel et al. supra). The expression library can also be screened with a polypeptide fragment, i.e., a portion of a polypeptide encoded by any of the above cited genes, by kinasing the polypeptide fragment with ^{32}phosphate. This method has been used previously by Yu et al. (1992 supra).

Other methods known to the art include the use of a complementation assay; a hybridization assay, e.g., for screening a genomic sample of DNA with a labelled nucleic acid; methods of DNA sequencing, and routine procedures for the maintenance of living cells, and the propagation, purification, and handling of DNA and proteins. (Sambrook et al. supra; Ausubel et al. supra; Miller et al. 1972 supra).

Other Embodiments

Other embodiments are within the following claims. For example, in addition to substantially full-length polypeptides, the present invention provides for agents that are biologically active fragments of the polypeptides. A pRB or transcription factor polypeptide

or fragment can be used as an agent of the invention if it exhibits a biological activity of a naturally occurring pRB or transcription factor. Such biological activities include the ability to bind, e.g., specifically bind, to a transcription factor (where the fragment comprises a portion of a pocket protein binding domain), or to pRB, (where the fragment comprises a portion of a transcription factor binding domain) as characterized herein. (The affinity of a fragment for pRB or for the appropriate transcription factor should be at least 1% of, more preferably at least 10% of, yet more preferably at least 50% of, and is most preferably at least 90%, the affinity of the naturally occurring proteins.).

The present invention also provides for analogs of polypeptides that inhibit or enhance the interaction of pRB with a cellular transcription factor, e.g., analogs of fragments of the binding domain of pRB or the factors. Analogs can differ from naturally occurring peptide fragments by amino acid sequence differences or by modifications which do not affect sequence, or by both.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro* chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences that have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

Included are peptides which have been modified so as to improve their resistance to proteolytic degradation

or to optimize solubility properties. Analogs can differ from naturally occurring pRB or transcription factor peptide fragments by alterations of their primary amino acid sequence. These peptides include genetic variants, both natural and induced. Induced mutants can be made by various techniques, e.g., by random mutagenesis of the encoding nucleic acids using irradiation or exposure to ethanemethylsulfate (EMS), or by site-specific mutagenesis or other techniques of molecular biology. See, Sambrook, Fritsch and Maniatis (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, hereby incorporated by reference. Also included are analogs which include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

The invention also provides for agents that are nucleic acid sequences, and purified preparations thereof, which encode the pRB or transcription factor polypeptides or peptide fragments described herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Bernardo Nadal-Ginard et al.

(ii) TITLE OF INVENTION: Control of Cell Proliferation

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: John W. Freeman
(B) REGISTRATION NUMBER: 29,066
(C) REFERENCE/DOCKET NUMBER: 00108/099001

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Asn Leu Phe Cys Ser Glu Glu Met Asp Ser Ser Asp Asp
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	5
(B) TYPE:	amino acid
(C) STRANDEDNESS:	N/A
(D) TOPOLOGY:	N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Leu Xaa Cys Xaa Glu
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	13
(B) TYPE:	amino acid
(C) STRANDEDNESS:	N/A
(D) TOPOLOGY:	N/A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asn Leu Phe Cys Ser Lys Glu Met Asp Ser Ser Asp Asp
1 5 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	15
(B) TYPE:	amino acid
(C) STRANDEDNESS:	N/A
(D) TOPOLOGY:	N/A

(ix) FEATURE:

(D) OTHER INFORMATION:	Xaa in locations 2 and 4 can be any ami acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Val Glu Ile Leu Arg Asn Ala Ile Arg Tyr Ile Glu Gly Leu
1 5 10 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	56
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGGATCCAAC ACCTGCTGCC TAACACCTGC TGCCTAACAC CTGCTGCCTA AGCTTG 56

CLAIMS

1. A method for identifying an agent that decreases undesired cell proliferation, said method comprising

(a) providing a system comprising a pocket protein and a factor that is required for terminal differentiation of a cell, said system being further characterized by conditions under which said pocket protein and said factor do not substantially interact;

(b) providing said candidate agent in said system; and

(c) measuring the interaction of said pocket protein with said factor, an increase of said interaction, relative to said interaction in the absence of said agent, indicating that said agent is capable of decreasing cell proliferation.

2. The method of claim 1, wherein said method further comprises

(d) providing a factor-specific deoxyribonucleic acid (DNA) sequence in said system, and

(e) determining the binding between said factor and said DNA sequence, an increase in said binding, relative to the amount of said binding in the absence of said agent, indicating that said agent is capable of decreasing cell proliferation.

3. The method of claim 1, wherein said system comprises a proliferating cell line, or an extract of said cell line, said method further comprising

(d) exposing the system to an antibody in the presence of said candidate agent, said antibody being capable of precipitating a complex between said pocket protein and said factor; and

(e) measuring the amount of said complex in said precipitate, an increase in the amount of said complex, relative to the amount of said complex precipitated from cells not exposed to said agent, indicating that said agent decreases cell proliferation.

4. The method of claim 1, further comprising

(d) providing a reporter gene in said system, the expression of said reporter gene being dependent on the interaction of said pocket protein with said factor; and

(e) measuring the level of expression of said reporter gene, an increase in said level, relative to the level of expression of said reporter gene in a system lacking said candidate agent, indicating that said candidate agent decreases cell proliferation.

5. The method of claim 4, wherein said system is a transgenic non-human mammal.

6. A method for identifying an agent that increases the interaction between a pocket protein and a factor required for terminal differentiation of a cell, said method comprising

(a) providing a proliferating cell line;

(b) allowing the cells of said cell line to differentiate in the presence of said candidate agent; and

(c) measuring the extent of differentiation of said cells, an increase in the extent of said differentiation, relative to the extent of said differentiation in cells not exposed to said agent, indicating that said agent decreases said interaction.

7. A method for identifying an agent that induces a terminally-differentiated cell to proliferate, said method comprising

(a) providing a system comprising a pocket protein and a factor that is required for terminal differentiation of a cell, said system being further characterized by conditions under which said pocket protein and said factor are capable of substantially interacting;

(b) providing said candidate agent in said system; and

(c) determining the interaction of said pocket protein with said factor, a decrease of said interaction, relative to said interaction in the absence of said agent, indicating that said candidate agent is capable of inducing a terminally differentiated cell to proliferate.

8. The method of claim 7, wherein said method further comprises

(d) providing a factor-specific deoxyribonucleic acid (DNA) sequence in said system; and

(e) determining the binding between said factor and said DNA sequence, a decrease in said binding, relative to the amount of said binding in the absence of said agent, indicating that said agent is capable of inducing a terminally differentiated cell to proliferate.

9. The method of claim 7, wherein said system is further characterized in being a differentiated cell line, or an extract of said cell line, said method further comprising

(d) exposing the system to an antibody in the presence of said candidate agent, said antibody being capable of precipitating a complex between said pocket protein and said factor; and

(e) measuring the amount of said complex in said precipitate, a decrease in the amount of said complex, relative to the amount of said complex precipitated from cells not exposed to said agent, indicating that said candidate agent is capable of inducing a terminally differentiated cell to proliferate.

10. The method of claim 7, wherein said system is further characterized in being a differentiated cell line, said interaction being determined by measuring the extent of differentiation of cells of said cell line, a decrease in the extent of said differentiation, relative to the extent of said differentiation in cells not exposed to said candidate agent, indicating that said candidate agent is capable of inducing a terminally differentiated cell to proliferate.

11. The method of claim 7, further comprising

(d) providing a growth factor in said system; and

(e) measuring the level of DNA synthesis in said system, an increase in the amount of said DNA synthesis, relative to the amount of DNA synthesis in a system lacking said candidate agent, indicating that said candidate agent is capable of inducing a terminally differentiated cell to proliferate.

12. The method of claim 7, further comprising

(d) providing a reporter gene in said system, the expression of said reporter gene being dependent on the interaction of said pocket protein with said factor; and

(e) measuring the level of expression of said reporter gene, a decrease in said level, relative to the level of expression of said reporter gene in a system lacking said candidate agent, indicating that said

candidate agent is capable of inducing a terminally differentiated cell to proliferate.

13. The method of claim 12, wherein said system is a transgenic non-human mammal.

14. A method for identifying an agent that mimics the effect of a factor required for terminal differentiation of a cell on a pocket protein, said method comprising

(a) providing a system, wherein said factor is absent or dysfunctional;

(b) providing an agent in said system; and

(c) observing whether said agent mimics the effect of said factor on said pocket protein.

15. The method of claim 14, wherein said agent mimics the effect of said factor by binding to said pocket protein.

16. The method of claim 14, wherein said system is a cultured cell line, and said observing comprises measuring the level of DNA replication in said cells, a decrease in DNA replication relative to the level of the DNA replication in cells not exposed to said agent indicating that said agent mimics the effect of said factor.

17. The method of claim 14, wherein said system is a cultured cell line and the pocket protein of the cells of said cultured cell line is absent or dysfunctional, further comprising introducing an isolated DNA into said cells, said DNA being substantially homologous to a gene encoding a functional pocket protein.

18. The method of claim 14, further comprising measuring the level of cell growth of said cells exposed to said agent, a decrease in the level of cell growth relative to the level of cell growth of cells not exposed to said agent indicating that said agent mimics the effect of said transcription factors.

19. The method of claim 14, further comprising measuring the amount of differentiation of said cells exposed to said agent, an increase in the amount of differentiation relative to the level of differentiation of cells not exposed to said agent indicating that said agent mimics the effect of said transcription factors.

20. A method for detecting a mutation in a gene encoding a pocket protein, said method comprising

(a) providing a system comprising a cellular factor required for terminal differentiation of a cell, said system being deficient in pocket protein function;

(b) providing a candidate pocket protein that bears a mutation in said system; and

(c) observing whether said pocket protein interacts with said factor, a decrease in said interaction, relative to the interaction of said factor with a wild-type pocket protein, indicating that the gene encoding said pocket protein bears said mutation.

21. The method of claim 20, wherein said method further comprises measuring cell proliferation.

22. A DNA molecule comprising the mutation of claim 20.

23. A method for detecting, in a human fetus or patient, a genetic predisposition to develop a neoplasm, said method comprising

(a) providing the DNA fragment of claim 22;

(b) exposing said DNA fragment to a nucleic acid sample obtained from said fetus or patient; and

(c) detecting hybridization of said DNA fragment to said nucleic acid sample.

24. A method for identifying a factor that interacts with a pocket protein so as to induce terminal differentiation of a cell, said method comprising co-isolating said pocket protein and said factor from said cell.

25. The method of claim 24, wherein said co-isolation is by immunoprecipitation.

26. A method for identifying a factor that interacts with a pocket protein so as to induce terminal differentiation of a cell, said method comprising

(a) providing a phage expression library specific for the cell-type of said cell,

(b) allowing said phage to form plaques,

(c) allowing said DNA of said library to be expressed,

(d) providing said pocket protein, and

(e) exposing said pocket protein to said plaques, binding of said pocket protein to said plaque indicating that the DNA of said plaque encodes said agent.

27. A method for identifying a factor that interacts with a pocket protein so as to induce terminal differentiation of a cell, said method comprising

(a) providing a complementation system that includes a selectable marker whose expression is under the control of a tissue-specific transcription factor,

(b) providing a pocket protein in said system; and

(c) observing the level of expression of said selectable marker, an increase in said level over background indicating that said system comprises said factor.

28. A method of decreasing undesired cell proliferation, said method comprising

administering to said cell an agent that interacts with a pocket protein in said cell so as to mimic the effect between said pocket protein and a factor required for terminal differentiation of said cell.

29. The method of claim 28, wherein said agent is said factor, or a fragment or analog thereof.

30. The method of claim 28, wherein said agent interacts with said pocket protein by binding to said pocket protein.

31. The method of claim 28, wherein said cell is a neoplastic cell.

32. The method of claim 28, wherein said cell is a non-neoplastic cell.

33. The method of claim 28, wherein said cell is in a mammal.

34. The method of claim 28, wherein said mammal is a human.

35. The method of claim 28, wherein said agent is a protein.

36. The method of claim 28, wherein said agent is a polypeptide or polypeptide analog.

37. The method of claim 28, wherein said pocket protein is retinoblastoma protein.

38. The method of claim 28, wherein said pocket protein is p107 protein.

39. The method of claim 28, wherein said agent causes a phosphate group to be removed from said pocket protein.

40. The method of claim 28, wherein said agent affects the expression of a pocket protein.

41. The method of claim 28, wherein said factor is a member of the MyoD family of proteins, or a fragment or analog thereof.

42. The method of claim 28, wherein said factor is a member of the MEF2 family of proteins, or a fragment or analog thereof.

43. The method of claim 28, wherein said factor is a member of the Mash family of proteins, or a fragment or analog thereof.

44. The method of claim 28, wherein said agent maintains the active conformation of said pocket protein.

45. The method of claim 28, wherein said agent maintains said active conformation by preventing phosphorylation of said pocket protein.

46. The method of claim 28, wherein said agent is a nucleic acid.

47. The method of claim 28, wherein said nucleic acid is administered by a gene therapy technique.

48. The method of claim 33, wherein said method further comprises the administration of a transgene that enhances the expression of a pocket protein in said mammal.

49. The method of claim 48, wherein said transgene is administered to a somatic cell of said mammal.

50. The method of claim 33, wherein said method is used to treat a neoplasm in a mammal.

51. A method of decreasing undesired cell proliferation, said method comprising

administering to said cell an agent that induces the expression of a gene product in said cell so as to mimic the effect between said pocket protein and a factor required for terminal differentiation of said cell.

52. A method of inducing the replication of a cell in a terminally differentiated state, said method comprising

administering to said cell an agent that interacts with a pocket protein in said cell so as to decrease the interaction between said pocket protein and a factor required for terminal differentiation in said cell.

53. The method of claim 52, wherein said cell is in a mammal.

54. The method of claim 52, wherein said agent is a protein.

55. The method of claim 52, wherein said agent is a polypeptide.

56. The method of claim 55, wherein said polypeptide is NLFCSEEMDSSDD (SEQ ID NO:1).

57. The method of claim 55, wherein said polypeptide is substantially homologous to a portion of an oncoprotein, or is an analog of a portion of an oncoprotein.

58. The method of claim 52, wherein said agent causes the addition of a phosphate group to said pocket protein.

59. The method of claim 52, wherein said agent binds the pocket domain of said pocket protein.

60. The method of claim 52, wherein said method is used to regenerate a tissue.

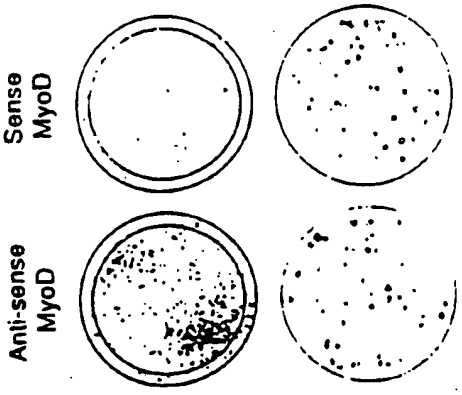
61. The method of claim 52, wherein said method is used to induce the formation of lymphatic cells.


A
T Ab
BrdU Ab
C2Ts11
C2K1
B
C2
C2Ts11
GM
DM
GM+pap
C
C2
C2Ts11
GM
DM
GM
DM
GM
pplB
plB

FIGURE 1

FIGURE 2

A



B

Cell Lines	Anti-sense MyoD Colony #	Sense MyoD Colony #	CFE % Reduction
U2OS	693	312	55
Saos	506	509	0

FIGURE 3

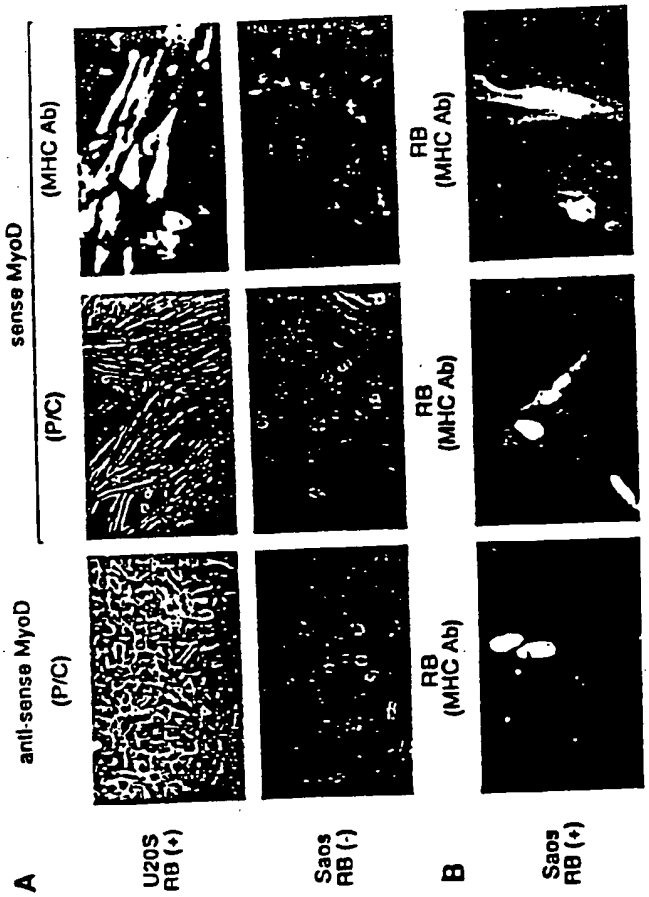

A
anti-sense MyoD (P/C)
sense MyoD (P/C)
sense MyoD (MHC Ab)
U2OS RB (+)
Saos RB (-)
B
RB (MHC Ab)
RB (MHC Ab)
RB (MHC Ab)
Saos RB (+)

FIGURE 4

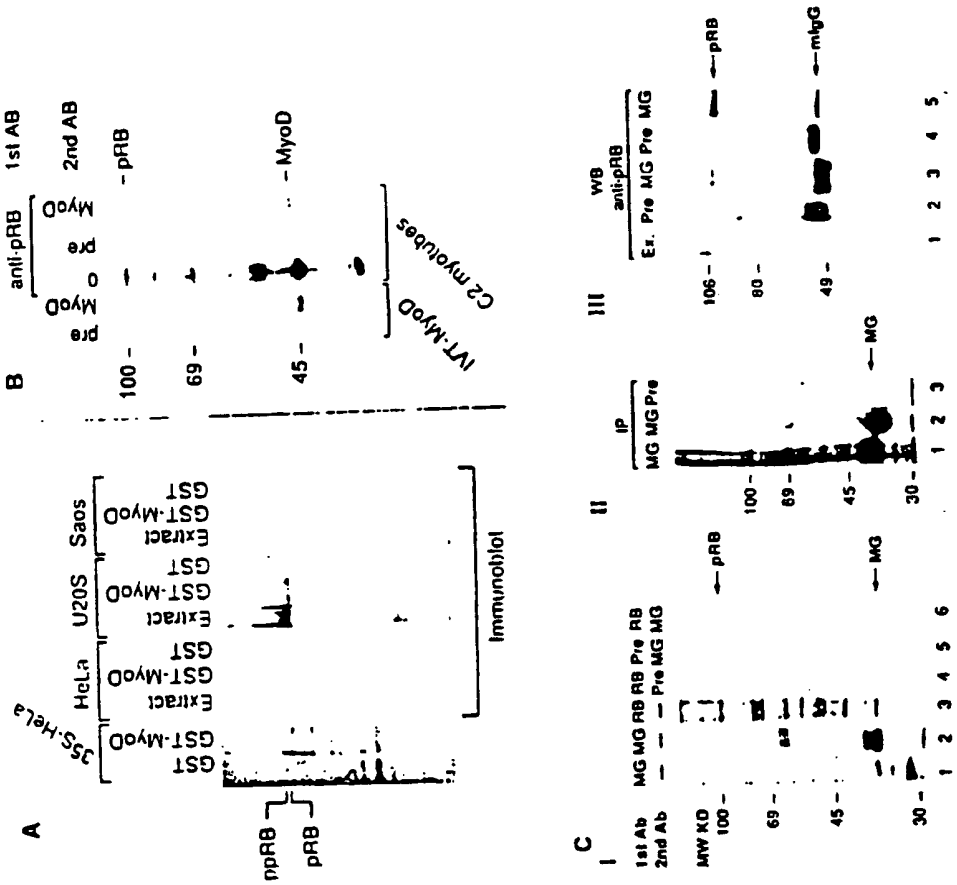

A
35S-HeLa
GST
GST-MyoD
Extract
HeLa
GST
GST-MyoD
Extract
U2OS
GST
GST-MyoD
Extract
Saos
GST
GST-MyoD
Extract
Immunoblot
pRB
ppRB
B
anti-pRB
pre MyoD
0
pre MyoD
1st AB
2nd AB
- pRB
- MyoD
100 -
69 -
45 -
IVT-MyoD
C2 myotubes
C
I
1st Ab
2nd Ab
MW XO
MG MG RB RB Pre RB
Pre MG MG
100 -
69 -
45 -
30 -
pRB
MG
1 2 3 4 5 6
II
IP
MG MG Pre
MG
100 -
69 -
45 -
30 -
1 2 3
III
WB
anti-pRB
Ex. Pre MG Pre MG
106 -
80 -
49 -
pRB
mIgG
1 2 3 4 5

FIGURE 5


A
kw Kd
100 -
69 -
MyoD → 45 -
30 -
Lysate
GST
GST-pRB (379-792)
GST-pRB (605-921)
GST-pRB (706 C-T)
B
kw Kd
100 -
69 -
45 -
30 -
pRB
Cyclin B
m pRB
m pRB/Sspl
m pRB/Dra III
Cyclin B
m pRB
m pRB/Sspl
m pRB/Dra III
MyoD
Lysate
GST-MyoD
C
RB proteins:
Binding to MyoD:
T/E1A-Binding Region
379
LZ
792
926
hu pRB
GST-pRB(379-792)
+++
GST- pRB(605-921)
+++
GST-pRB(379-928;706 C → F)
±
Dra III
656
Ssp I
760
921
m pRB
+++
m pRB/Sspl (1-760)
±
m pRB/Dra III (1-656)
-

FIGURE 6


B
MyoD proteins:
Binding to RB:
bHLH
1
318
MyoD
DM:3-56
DM:63-99
DM:102-135
B2proB3
DM:174-318
DM:127-318
+ + + - + + -
A
Kd
46-
30-
GST
GST-pRB(605-921)
MyoD
MyoD
DM:3-56
DM:63-99
DM:102-135
B2proB3
DM:174-318
DM:127-318

FIGURE 7


B
kD
69-
46-
E2-2
E12
MyoD
E2-2
E12
MyoD
Lysate
GST-pRB(373-928)
A
m pRB
m pRB+T pep
m pRB+K1 pep
pRB
GST-MyoD

FIGURE 8

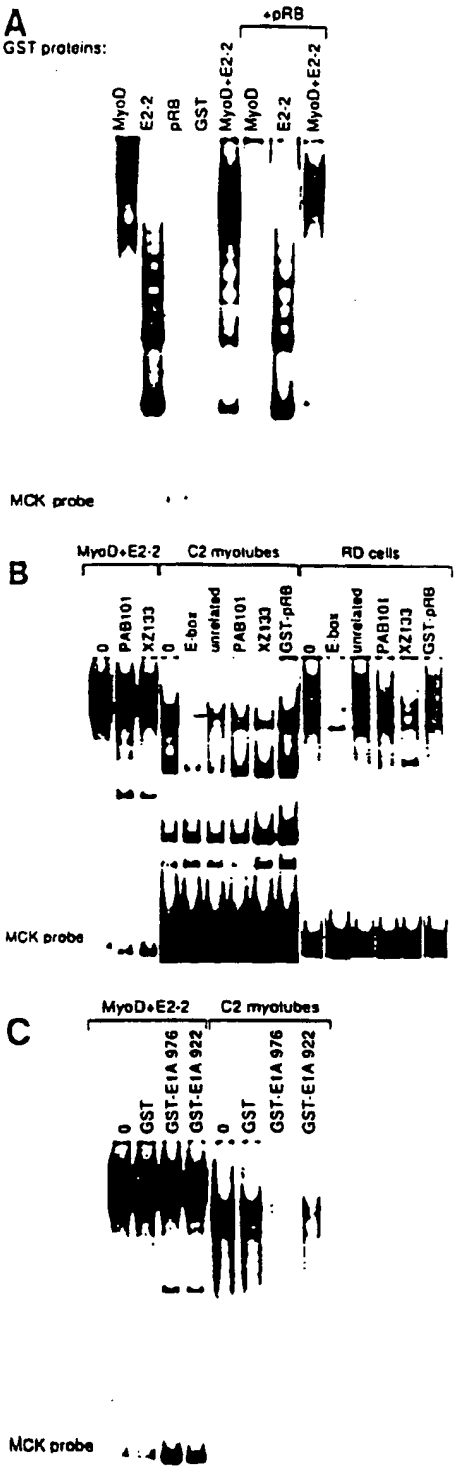
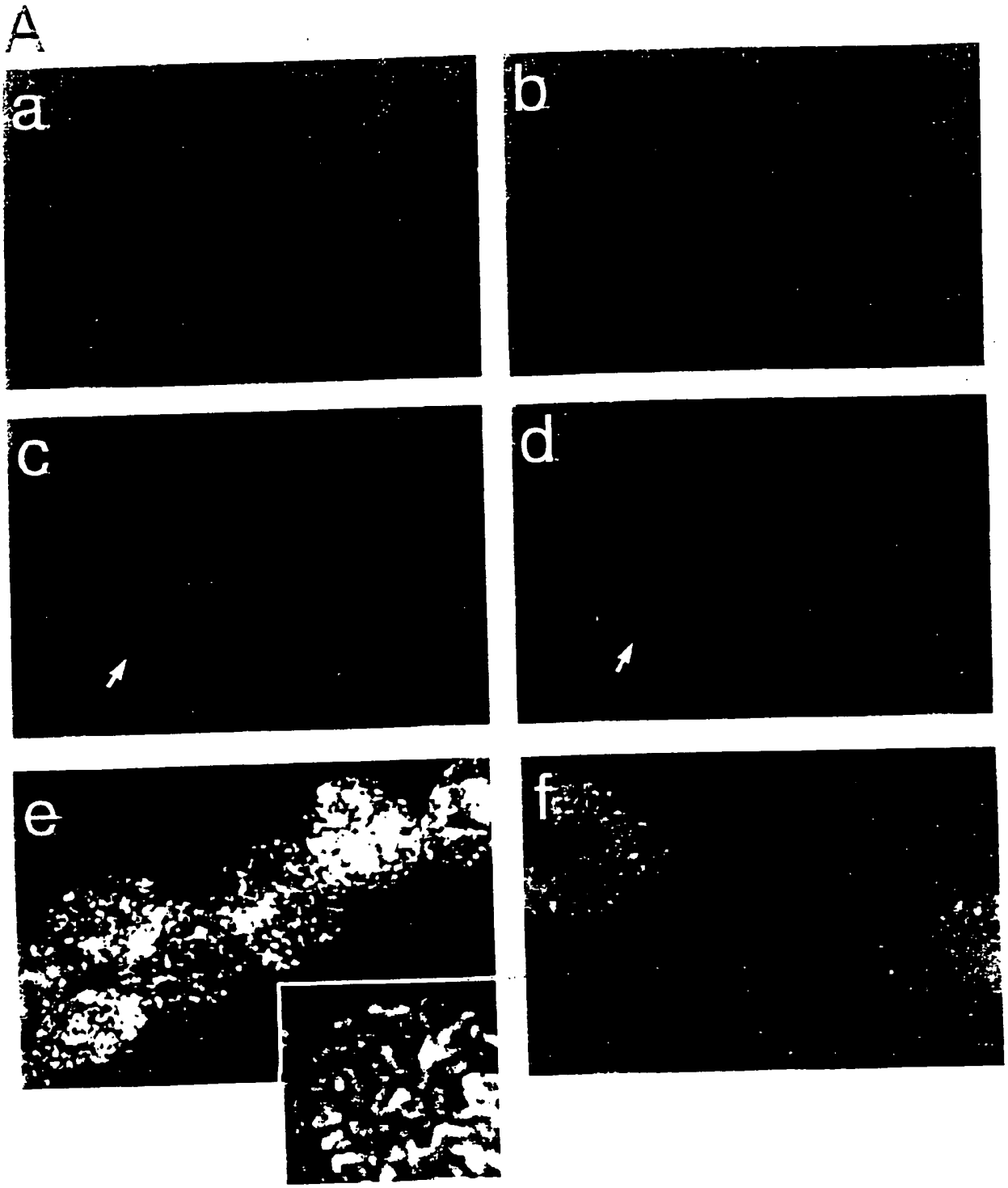

A
GST proteins:
MyoD
E2-2
pRB
GST
MyoD+E2-2
MyoD
E2-2
+pRB
MyoD+E2-2
MCK probe
B
MyoD+E2-2
C2 myotubes
RD cells
0
PAB101
XZ133
0
E-box
unrelated
PAB101
XZ133
GST-pRB
0
E-box
unrelated
PAB101
XZ133
GST-pRB
MCK probe
C
MyoD+E2-2
C2 myotubes
0
GST
GST-E1A 976
GST-E1A 922
0
GST
GST-E1A 976
GST-E1A 922
MCK probe

Figure 9A



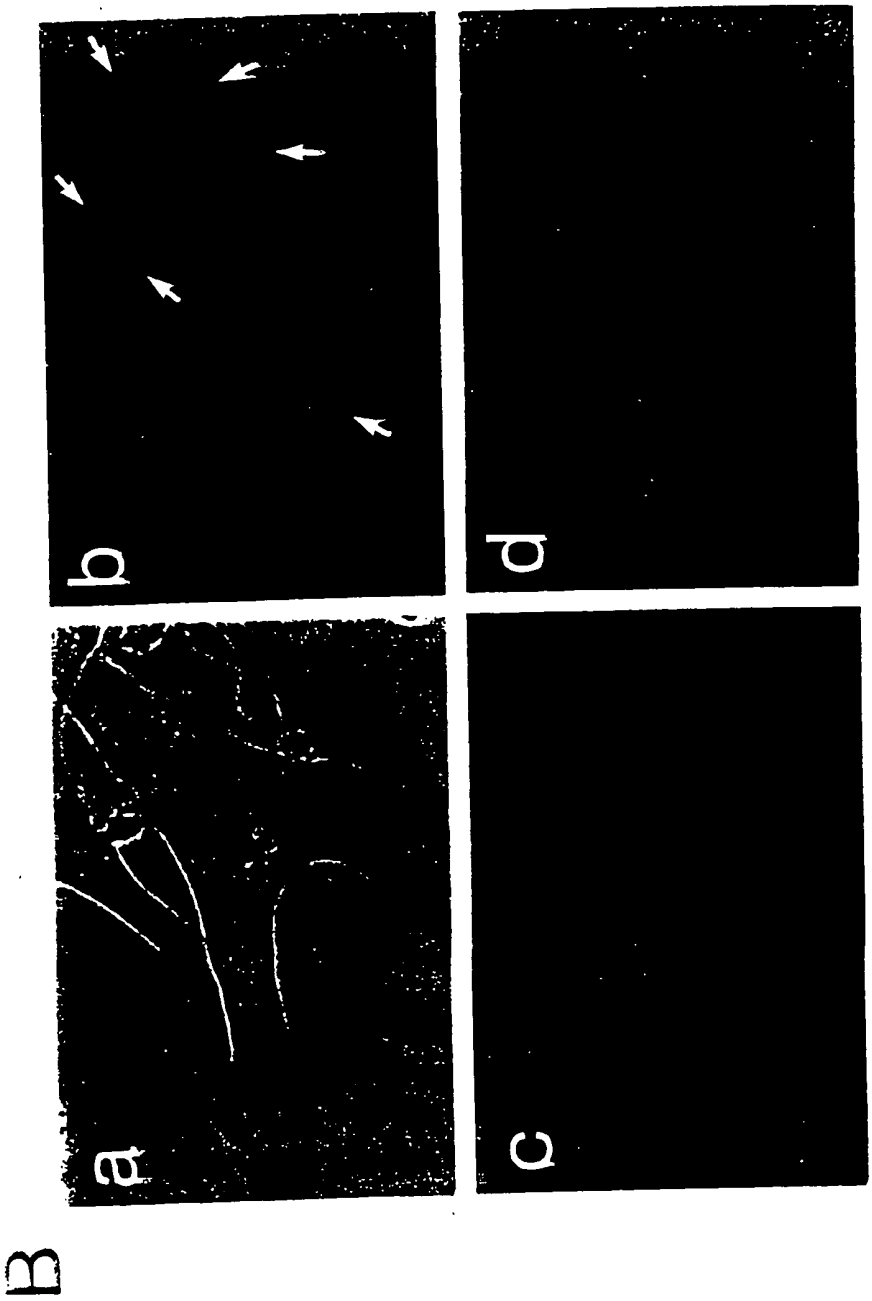


Figure 9B

Figure 10

MYOGENIC FACTOR SEQUENCES THAT INTERACT WITH THE POCKET PROTEINS

	BASIC REGION	JOINT	HELIX
MyoD	K R K T T N A D R R K A A T M R E R R R	L S K V	N E A F E T L K R
Myogenin	K R K S V S V D R R R A A T L R E K R R	L K K V	N E A F E A L K R
myf-5	K R K S T T M D R R K A A T M R E R R R	L S K V	N Q A R E T L K R
MRF-4	K R K S A P T D R R K A A T M R E R R R	L S K V	N E A F E T L K R

MASH SEQUENCES THAT INTERACT WITH THE POCKET PROTEINS

MASH1	R R - - - N E R E R N R	V K L V	N L G F A T L R E
MASH2	R R - - - N E R N R N R	V K L V	N L G F Q A L R Q

Figure 11

MEF2 FACTOR SEQUENCES THAT INTERACT WITH POCKET PROTEINS

	..K.....I. ....R..T.. KR..G..KK. ..L..L....		DMD.VLLKYT EY.EPHESRT NSDI.E		
Consensus	rgKi.ikrIe nktnRqvTfs KRrnGlmKKa yeLsvLcdae .aliifsst. klyeyasp		DMDkVLLKYT EYnEPHESRT NSDIvE		
	Helix1 aa 20-33		Helix2 aa 60-69		
hMEF2C	RKKIQITRIM DERNRQVTFT KRKFGLMKKA YELSVLCDCE IALIIFNSTN KLFQYAST	-	DMDKVLLKYT EYNEPHESRT NSDIVE	-	
MEF2	RKKIQITRIM DERNRQVTFT KRKFGLMKKA YELSVLCDCE IALIIFNSSN KLFQYAST	57/58	DMDKVLLKYT EYNEPHESRT NSDIVE	26/26	
xMEF2	RKKIQISRIL DQRNRQVTFT KRKFGLMKKA YELSVLCDCE IALIIFNSAN RLFQYAST	53/58	DMDRVLLKYT EYSEPHESRT NTDILE	24/26	
hMEF2D	RKKIQIQRIM DERNRQVTFT KRKFGLMKKA YELSVLCDCE IALIIFNHSN KLFQYAST	55/58	DMDKVLLKYT EYNEPHESRT NADIIE	24/26	

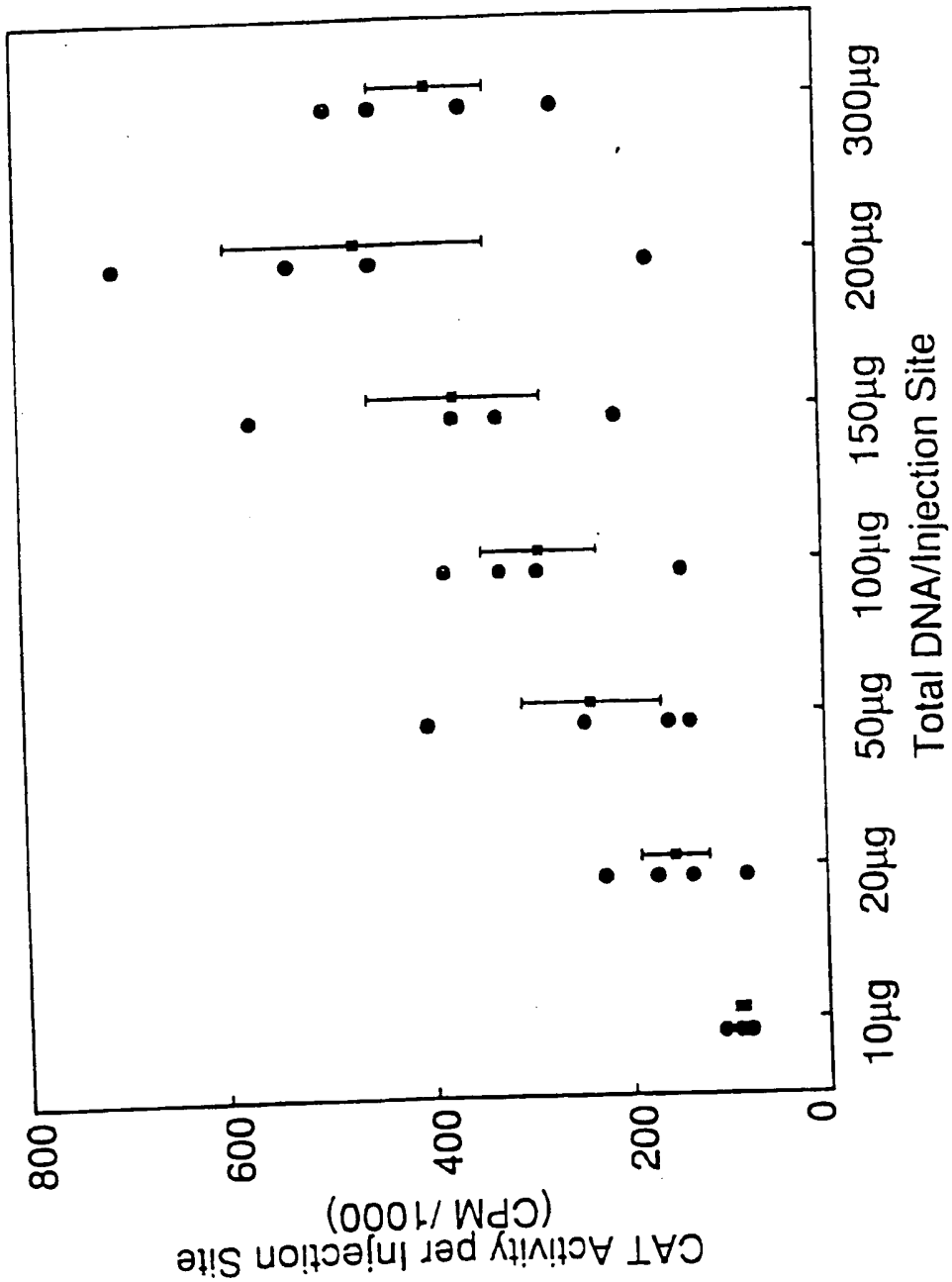

CAT Activity per Injection Site
(CPM /1000)
800
600
400
200
0
10µg
20µg
50µg
100µg
150µg
200µg
300µg
Total DNA/Injection Site

Figure 12

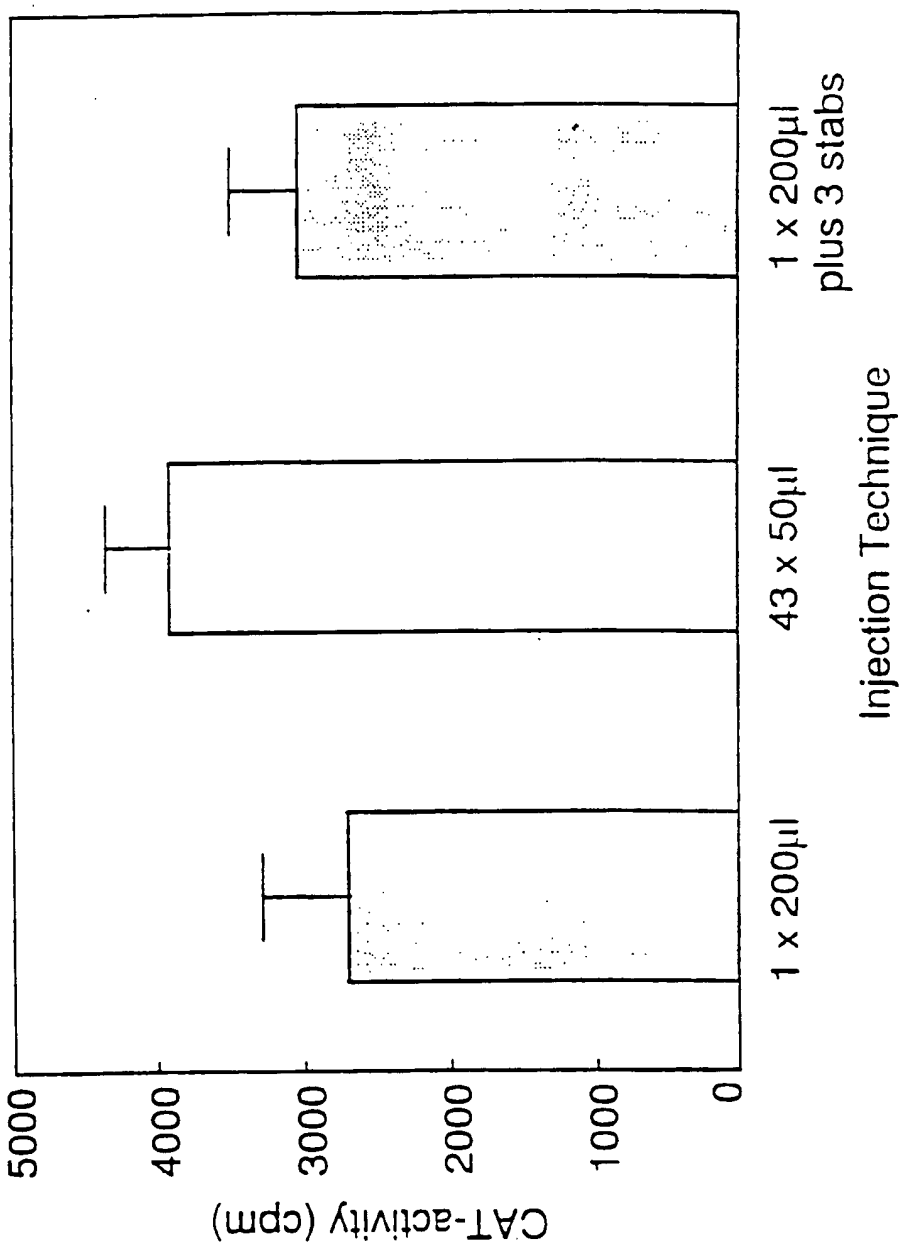

CAT-activity (cpm)
5000
4000
3000
2000
1000
0
1 x 200µl
43 x 50µl
1 x 200µl
plus 3 stabs
Injection Technique

Figure 13

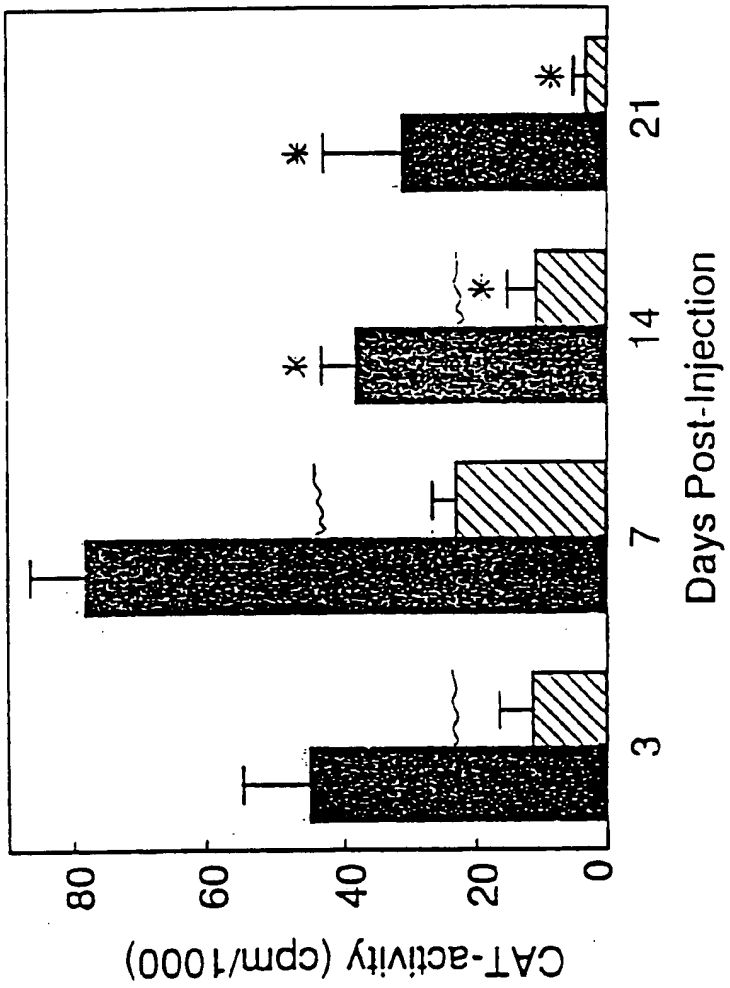

CAT-activity (cpm/1000)
0
20
40
60
80
3
7
14
21
Days Post-Injection
*
*
*
*

Figure 14

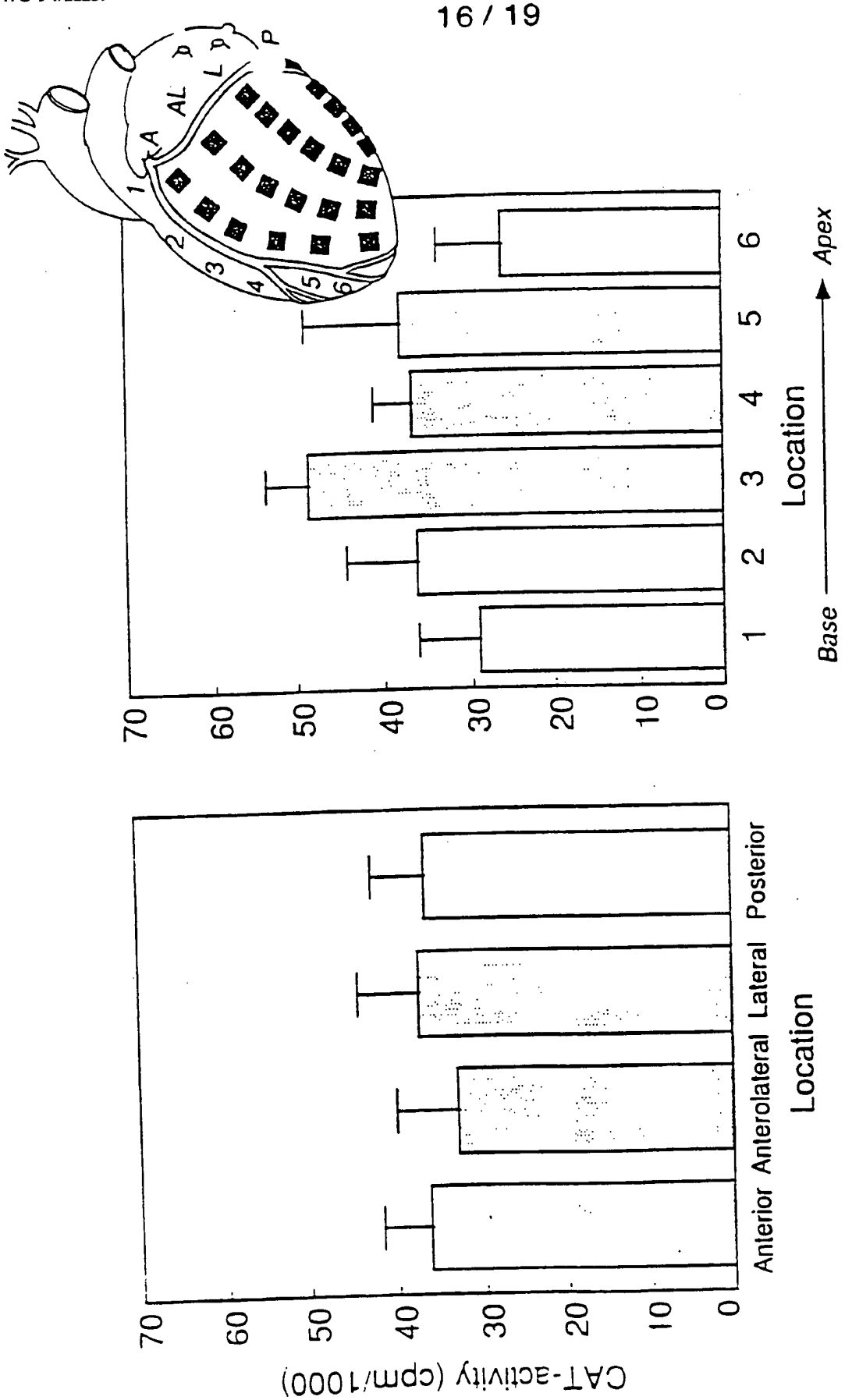

A
AL
L
P
1
2
3
4
5
6
CAT-activity (cpm/1000)
0
10
20
30
40
50
60
70
Anterior
Anterolateral
Lateral
Posterior
Location
Base
Apex
Location

Figure 15

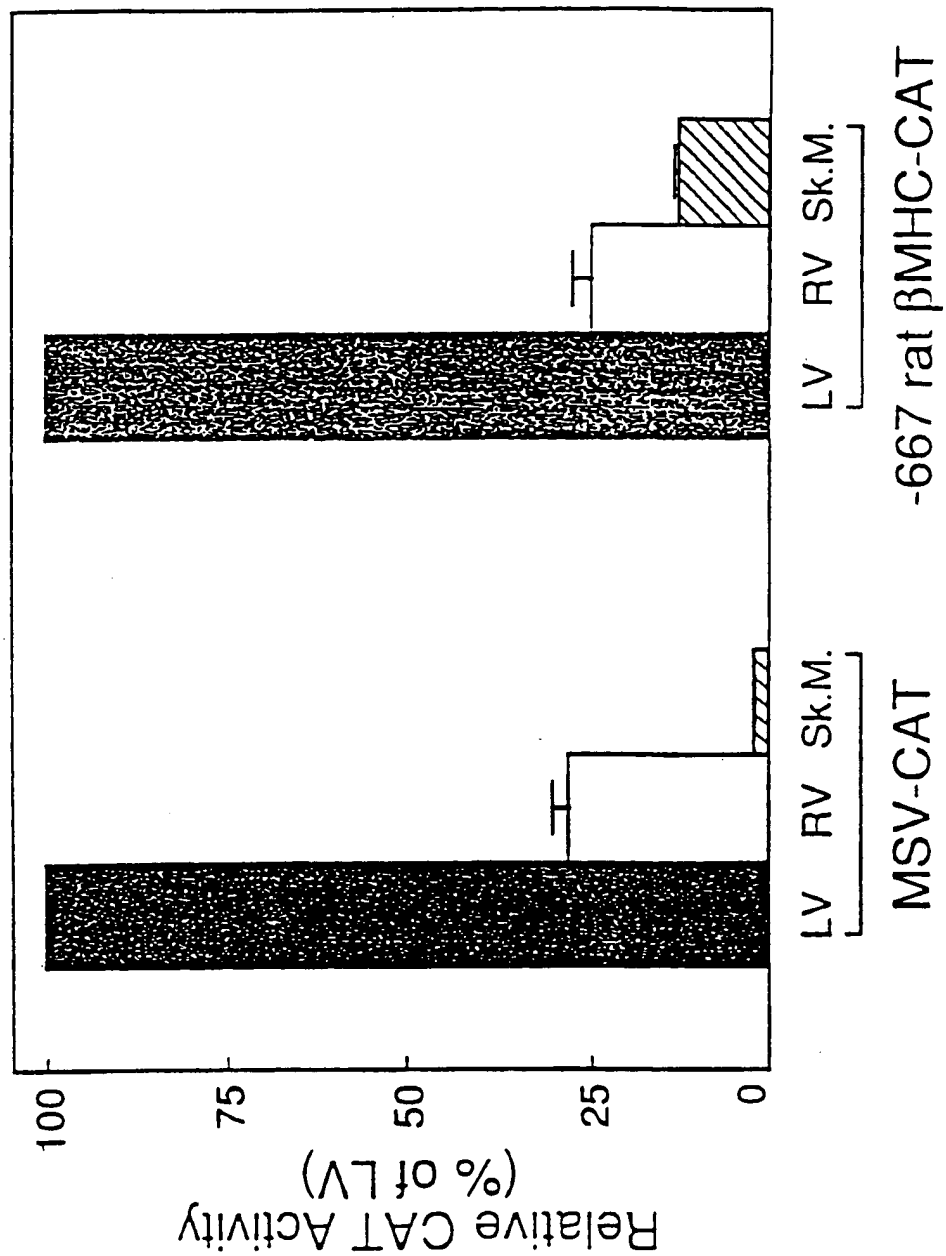
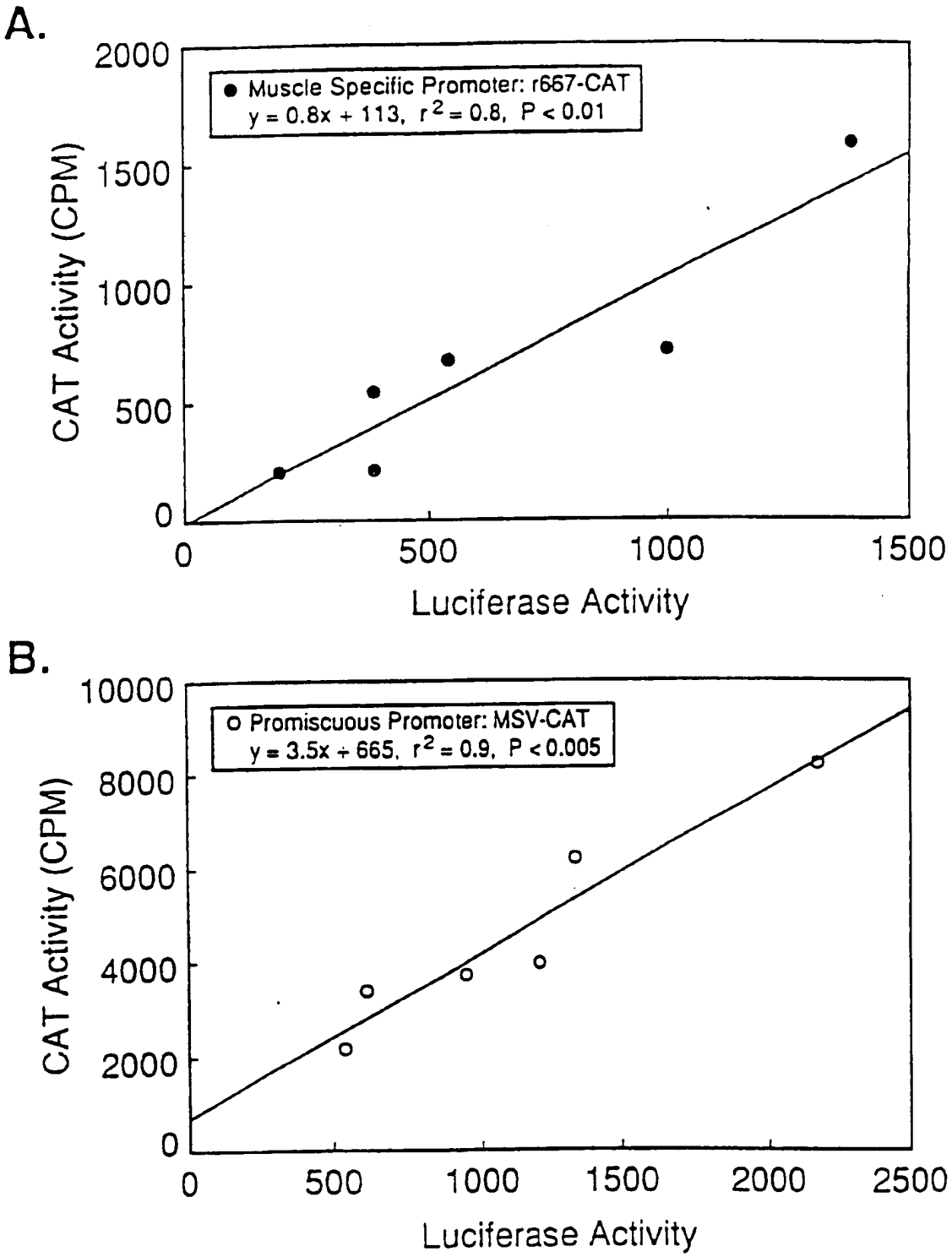

Relative CAT Activity
(% of LV)
100
75
50
25
0
LV RV Sk.M.
MSV-CAT
LV RV Sk.M.
-667 rat βMHC-CAT

Figure 16

Figure 17


A.
Muscle Specific Promoter: r667-CAT
y = 0.8x + 113, r2 = 0.8, P < 0.01
CAT Activity (CPM)
Luciferase Activity
0
500
1000
1500
2000
B.
Promiscuous Promoter: MSV-CAT
y = 3.5x + 665, r2 = 0.9, P < 0.005
CAT Activity (CPM)
Luciferase Activity
0
2000
4000
6000
8000
10000
2500

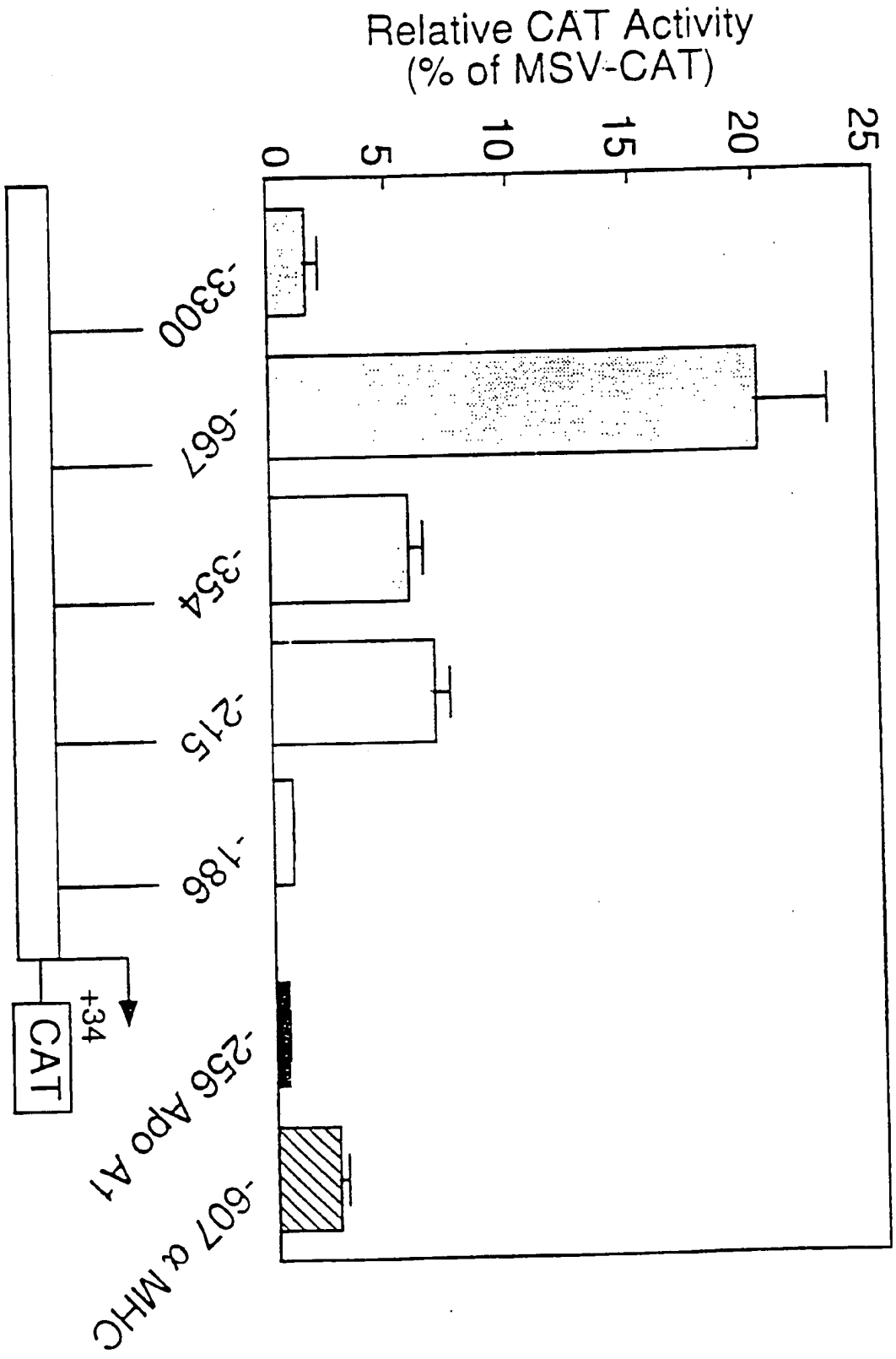

Relative CAT Activity
(% of MSV-CAT)
0
5
10
15
20
25
-330
-667
-354
-215
-186
+34
CAT
-256 Apo A1
-607 αMHC

Figure 18

INTERNATIONAL SEARCH REPORT

Ir. ational application No.
PCT/US94/01499

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00, 17/00; C12N 15/00; C12Q 1/00, 1/68; G01N 33/58

US CL :435/6, 7.1, 7.2, 7.21, 7.6, 172.3; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.2, 7.21, 7.6, 172.3; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONCOGENE, Volume 7, Issued 1992, M. Dobbelstein et al., "Intracistronic complementation reveals a new function of SV40 T antigen that co-operates with Rb and p53 to stimulate DNA synthesis in quiescent cells", pages 837-847, see entire document.	1-4 and 6-12
Y	ONCOGENE, Volume 7, issued 1992, J. Bartek et al., "Cellular localization and T antigen binding of the retinoblastoma protein", pages 101-108, see entire article.	1-4 and 6-12
Y	NATURE, Volume 334, Issued 1988, P. Whyte et al., "Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product", pages 124-129, see entire article.	1-4 and 6-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 MAY 1994	Date of mailing of the international search report 16 MAY 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Brian R. Stanton Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US94/01499

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 243, Issued 17 February 1989, N. Dyson et al., "The Human Papilloma Virus-16 E7 Oncoprotein Is Able to Bind to the Retinoblastoma Gene Product", pages 934-937, see entire article.	1-4 and 6-12
Y	SCIENCE, Volume 243, Issued 17 February 1989, J.M. Horowitz et al., "Point Mutational Inactivation of the Retinoblastoma Antioncogene", pages 937-940, see entire article.	1-4 and 6-12
Y	SCIENCE, Volume 247, Issued 09 February 1990, R. Bookstein et al., "Suppression of Tumorigenicity of Human Prostate Carcinoma Cells by Replacing a Mutated *RB* Gene", pages 712-715, see entire article.	1-4 and 6-12
P,X	CELL, Volume 72, Issued 12 February 1993, W. Gu et al., "Interaction of Myogenic Factors and the Retinoblastoma Protein Mediates Muscle Cell Commitment and Differentiation", pages 309-324, see entire document.	1-4 and 6-12
Y	CELL, Volume 54, Issued 15 July 1988, J.A. DeCaprio et al., "SV40 Large T Antigen Forms a Specific Complex with the Product of the Retinoblastoma Susceptibility Gene Product", pages 275-283, see entire article.	1-4 and 6-12
Y	CELL, Volume 66, Issued 20 September 1991, M.E. Ewen et al., "Molecular Cloning, Chromosomal Mapping, and Expression of the cDNA for p107, a Retinoblastoma Gene Product-Related Protein", pages 1155-1164, see entire article.	1-4 and 6-12
Y	CELL, Volume 56, Issued 13 January 1989, J.W. Ludlow et al., "SV40 Large T Antigen Binds Preferentially to an Underphosphorylated Member of the Retinoblastoma Susceptibility Gene Product Family", pages 57-65, see entire article.	1-4 and 6-12
Y	CELL, Volume 56, Issued 13 January 1989, P. Whyte et al., "Cellular Targets for Transformation by the Adenovirus E1A Proteins", pages 67-75, see entire article.	1-4 and 6-12
Y	CELL, Volume 60, Issued 09 February 1990, J.W. Ludlow et al., "The Retinoblastoma Susceptibility Gene Product Undergoes Cell Cycle-Dependent Dephosphorylation and Binding to and Release from SV40 Large T", pages 387-396, see entire article.	1-4 and 6-12

INTERNATIONAL SEARCH REPORT

Int .tional application No.
PCT/US94/01499

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF VIROLOGY, Volume 64, Number 3, Issued March 1990, N. Dyson et al., "Large T Antigens of Many Polyomaviruses Are Able To Form Complexes with the Retinoblastoma Protein", pages 1353-1356, see entire article.	1-4 and 6-12
Y	JOURNAL OF VIROLOGY, Volume 65, Number 6, Issued June 1991, L. Resnick-Silverman et al., "Retinoblastoma Protein and Simian Virus 40-Dependent Immortalization of Human Fibroblasts", pages 2845-2852, see entire article.	1-4 and 6-12
Y	THE EMBO JOURNAL, Volume 9, Number 4, Issued 1990, Q. Hu et al., "The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations", pages 1147-1155, see entire article.	1-4 and 6-12

INTERNATIONAL SEARCH REPORT

Int. tional application No.
PCT/US94/01499

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4 and 6-12

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CA, Medline, Biosis, Embase
Search Terms: myo?; rb; p94; p97; p100; p107; p300; pocket; protein?; antigen?; ag; schneider?/au; mahdavi?/au; gu?/au; nadal ginard?/au

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-4 and 6-12, drawn to in vitro methods of measuring the effect of an agent on the interaction between a pocket protein and a factor.

II. Claims 1-13, drawn to in vivo methods of measuring the effect of an agent on the interaction between a pocket protein and a factor.

III. Claims 14-19, 24, 25 and 27, drawn to in vitro methods of identifying agents that interact with pocket proteins.

IV. Claims 14, 15, 18, 19, 24, 25 and 27, drawn to in vivo methods of identifying agents that interact with pocket proteins.

V. Claims 20-22, drawn to in vitro methods of detecting pocket factor mutants in eukaryotic systems and DNA encoding pocket factors.

VI. Claims 20-23, drawn to in vivo methods of detecting pocket factor mutants in eukaryotic systems and methods of detecting genetic defects.

VII. Claim 26, drawn to methods of detecting pocket factor mutants in prokaryotic systems.

VIII. Claims 28-32, 35-39, 44 and 45, drawn to in vitro methods of altering cellular proliferation using Rb proteins.

IX. Claims 28-32, 35, 36, 40, and 44, drawn to in vitro methods of altering cellular proliferation using transcription factors.

X. Claims 28-32, 35, 36, 41, and 44, drawn to in vitro methods of altering cellular proliferation using MyoD protein.

XI. Claims 28-32, 35, 36, 42, and 44, drawn to in vitro methods of altering cellular proliferation using MEF2 protein.

XII. Claims 28-32, 35, 36, 43, and 44, drawn to in vitro methods of altering cellular proliferation using MASH protein.

XIII. Claims 28-32, 35, 36, 44, 46 and 47, drawn to in vitro methods of altering cellular proliferation using DNA protein.

XIV. Claims 28-34, 35-39, 44, 45, and 48-50, drawn to in vivo methods of altering cellular proliferation using Rb proteins.

XV. Claims 28-34, 35, 36, 40, 44 and 48-50, drawn to in vivo methods of altering cellular proliferation using transcription factors.

XVI. Claims 28-34, 35, 36, 41, 44 and 48-50, drawn to in vivo methods of altering cellular proliferation using MyoD protein.

XVII. Claims 28-34, 35, 36, 42, 44 and 48-50, drawn to in vivo methods of altering cellular proliferation using MEF2 protein.

XVIII. Claims 28-34, 35, 36, 43, 44 and 48-50, drawn to in vivo methods of altering cellular proliferation using

MASH protein.

XIX. Claims 28-34, 35, 36, 44, 46 and 47-50, drawn to in vivo methods of altering cellular proliferation using DNA protein.

XX. Claim 51, drawn to methods of altering cellular proliferation by altering gene expression.

XXI. Claims 52-61, drawn to in vitro methods of inducing cellular replication using proteinaceous agents.

XXII. Claims 52-61, drawn to in vivo methods of inducing cellular replication using proteinaceous agents.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions are distinct, each from the other because of the following reasons:

The inventions of Groups I-III and V-VII are distinct, one from the other because they are drawn to materially different methods requiring non-coextensive considerations. Note that PCT Rule 13.2 does not provide for multiple methods within a single inventive concept. For example, the methods of Group I are directed towards the elaboration of agents which affect the interaction between the a factor and a pocket protein. The methods of group II are directed towards the elaboration of agents which interact with the pocket protein alone. Therefore, the agents would have been expected to have functioned in materially different fashions, one working on a complex and one interacting with a particular protein. The methods of Group III are directed towards the elaboration of pocket protein mutants and therefore requires consideration of protein mutants and determination of what would be considered to be a pocket protein. The methods of Group V require consideration of genetic disorders and such considerations are not required for any of the other groups. The methods of Groups III and VI require divergent considerations because in the case of the invention of Group III, eukaryotic systems are employed whereas in the invention of Group VI, prokaryotic systems are used. These disparate recombinant systems involve different considerations related to the fundamental biologically differences between prokaryotic and eukaryotic organisms. Methods of the invention of Group VI are based upon the alteration of gene expression and therefore require consideration of gene regulation which is not necessarily required for consideration of the other groups of inventions. The methods of Group VII are directed towards the induction of cellular replication and require consideration of factors involved in the regulation of cell proliferation and do not necessarily involve the analysis of the interaction of any particular sets of factors as required, for example, for consideration of the invention of Group I.

The DNA of group IV is distinct from the methods of any of inventions I, II, and VI-VII because the DNA is not required for those methods and is therefore not necessarily related.

The *in vitro* and *in vivo* distinction of the claimed methods of groups VIII-XIX, XXI and XXII as elaborated above represent distinct inventions because they represent materially different processes which require disparate considerations. For example, *in vivo* methods require consideration of routes of administration, determination of the form of the agents administered, assays for determination of *in vivo* efficacy, stability within the *in vivo* milieu, etc. and none of these considerations are required for consideration of *in vitro* methods.

The inventions of groups VIII-XXII wherein protein versus nucleic acid (DNA) agents are distinct one from the other because they require materially different considerations. For example, use of DNA requires consideration of transcriptional and translational control elements which are not required for the use of proteinaceous agents. The inventions employing different species of proteins represent distinct inventions because they employ dissimilar proteins which would have been expected to have had unique and non-coextensive properties. The search of the non-patent literature would therefore require analysis of art directed towards each particular protein type.

For the reasons elaborated above, the various inventions as outlined above lack a special technical feature within the meaning of PCT Rule 13.2 and therefore unity of invention is lacking.